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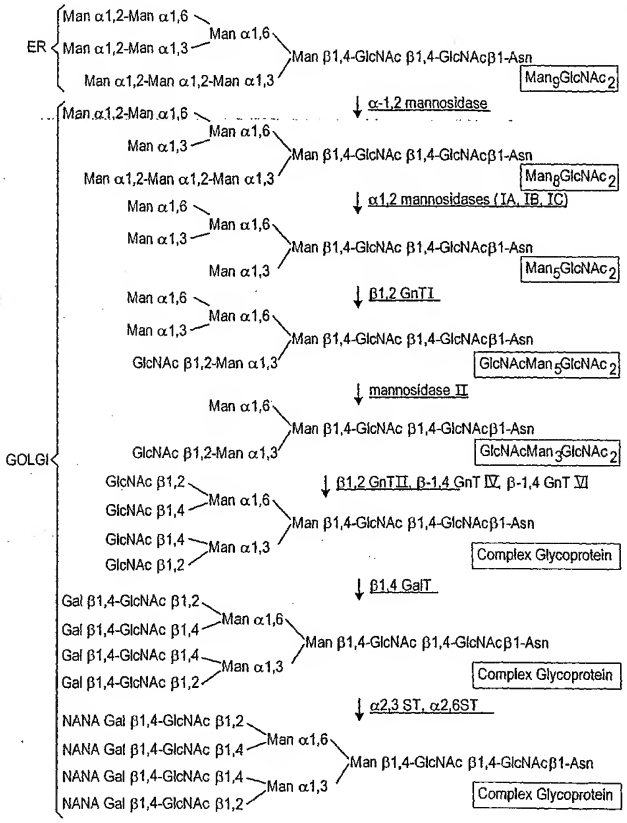

(57) Abstract: The present invention relates to eukaryotic host cells having modified oligosaccharides which may be modified further by heterologous expression of a set of glycosyltransferases, sugar transporters and mannosidases to become host-strains for the production of mammalian, e.g., human therapeutic glycoproteins. The invention provides nucleic acid molecules and combinatorial libraries which can be used to successfully target and express mammalian enzymatic activities such as those involved in glycosylation to intracellular compartments in a eukaryotic host cell. The process provides an engineered host cell which can be used to express and target any desirable gene(s) involved in glycosylation. Host cells with modified oligosaccharides are created or selected. N-glycans made in the engineered host cells have a Man5GlcNAc2 core structure which may then be modified further by heterologous expression of one or more enzymes, e.g., glycosyltransferases, sugar transporters and mannosidases, to yield human-like glycoproteins. For the production of therapeutic proteins, this method may be adapted to engineer cell lines in which any desired glycosylation structure may be obtained.

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COMBINATORIAL DNA LIBRARY FOR PRODUCING MODIFIED *N*-GLYCANS IN LOWER EUKARYOTES

STATEMENT AS TO FEDERALY SPONSORED RESEARCH

[0001] This invention was funded, at least in part, with a government grant from the National Institutes of Health (NIH Phase I Grant no. 1R43GM66690-1) and a grant from the Department of Commerce, NIST-ATP Cooperative Agreement Number 70NANB2H3046. The Unites States government may therefore have certain rights in this invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to United States Application Serial No. 10/371,877 which is a continuation-in-part of United States Application Serial No. 09/892,591, filed June 27, 2001, in which priority is claimed to U.S. Provisional Application Serial No. 60/214,358, filed June 28, 2000, U.S. Provisional Application No. 60/215,638, filed June 30, 2000, and U.S. Provisional Application No. 60/279,997, filed March 30, 2001; each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention is directed to methods and compositions by which non-human eukaryotic host cells, such as fungi or other eukaryotic cells, can be genetically modified to produce glycosylated proteins (glycoproteins) having patterns of glycosylation similar to those of glycoproteins produced by animal cells, especially human cells, which are useful as human or animal therapeutic agents.

BACKGROUND OF THE INVENTION

Glycosylation Pathways in Humans and Lower Eukaryotes

[0004] After DNA is transcribed and translated into a protein, further post-translational processing involves the attachment of sugar residues, a process known as glycosylation. Different organisms produce different glycosylation enzymes (glycosyltransferases and glycosidases), and have different substrates (nucleotide sugars) available, so that the glycosylation patterns as well as composition of the individual oligosaccharides, even of the same protein, will be different depending on the host system in which the particular protein is being expressed. Bacteria typically do not glycosylate proteins, and if so only in a very unspecific manner (Moens and Vanderleyden, 1997 *Arch Microbiol.* 168(3):169-175). Lower eukaryotes such as filamentous fungi and yeast add primarily mannose and mannosylphosphate sugars. The resulting glycan is known as a "high-mannose" type glycan or a mannan. Plant cells and insect cells (such as Sf9 cells) glycosylate proteins in yet another way. By contrast, in higher eukaryotes such as humans, the nascent oligosaccharide side chain may be trimmed to remove several mannose residues and elongated with additional sugar residues that typically are not found in the *N*-glycans of lower eukaryotes. See, e.g., R.K. Bretthauer, et al. *Biotechnology and Applied Biochemistry*, 1999, 30, 193-200; W. Martinet, et al. *Biotechnology Letters*, 1998, 20, 1171-1177; S. Weikert, et al. *Nature Biotechnology*, 1999, 17, 1116-1121; M. Malissard, et al. *Biochemical and Biophysical Research Communications*, 2000, 267, 169-173; Jarvis, et al., *Current Opinion in Biotechnology*, 1998, 9:528-533; and M. Takeuchi, 1 *Trends in Glycoscience and Glycotechnology*, 1997, 9, S29-S35.

[0005] Synthesis of a mammalian-type oligosaccharide structure begins with a set of sequential reactions in the course of which sugar residues are added and removed while the protein moves along the secretory pathway in the host organism. The enzymes which reside along the glycosylation pathway of the host organism or cell determine the resulting glycosylation patterns of secreted proteins. Thus, the resulting glycosylation pattern of proteins expressed in lower eukaryotic host cells differs substantially from the glycosylation pattern of proteins expressed in higher eukaryotes such as humans and other mammals (Bretthauer, 1999). The structure of a typical fungal *N*-glycan is shown in **Fig. 1A**.

[0006] The early steps of human glycosylation can be divided into at least two different phases: (i) lipid-linked $Glc_3Man_9GlcNAc_2$ oligosaccharides are assembled by a sequential set of reactions at the membrane of the endoplasmic reticulum (ER) and (ii) the transfer of this oligosaccharide from the lipid anchor dolichyl pyrophosphate onto *de novo* synthesized protein. The site of the specific transfer is defined by an asparagine (Asn) residue in the sequence Asn-Xaa-Ser/Thr where Xaa can be any amino acid except proline (Gavel, 1990). Further processing by glucosidases and mannosidases occurs in the ER before the nascent glycoprotein is transferred to the early Golgi apparatus, where additional mannose residues are removed by Golgi specific alpha (α)-1,2-mannosidases. Processing continues as the protein proceeds through the Golgi. In the medial Golgi, a number of modifying enzymes, including *N*-acetylglucosaminyl Transferases (GnTI, GnTII, GnTIII, GnTIV and GnTV), mannosidase II and fucosyltransferases, add and remove specific sugar residues. Finally, in the trans-Golgi, galactosyltranferases (GalT) and sialyltransferases (ST) produce a glycoprotein structure that is released from the Golgi. It is this structure, characterized by bi-, tri- and tetra-antennary structures, containing galactose, fucose, N-acetylglucosamine and a high degree of terminal sialic acid, that gives glycoproteins their human characteristics. The structure of a typical human *N*-glycan is shown in **Fig. 1B**.

[0007] In nearly all eukaryotes, glycoproteins are derived from a common lipid-linked oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-dolichol-pyrophosphate. Within the endoplasmic reticulum, synthesis and processing of dolichol pyrophosphate bound oligosaccharides are identical between all known eukaryotes.

However, further processing of the core oligosaccharide by fungal cells, e.g., yeast, once it has been transferred to a peptide leaving the ER and entering the Golgi, differs significantly from humans as it moves along the secretory pathway and involves the addition of several mannose sugars.

[0008] In yeast, these steps are catalyzed by Golgi residing mannosyl-transferases, like Och1p, Mnt1p and Mnn1p, which sequentially add mannose sugars to the core oligosaccharide. The resulting structure is undesirable for the production of human-like proteins and it is thus desirable to reduce or eliminate mannosyltransferase activity. Mutants of *S.cerevisiae*, deficient in mannosyl-transferase activity (for example *och1* or *mnn9* mutants) have been shown to be non-lethal and display reduced mannose content in the oligosaccharide of yeast glycoproteins. Other oligosaccharide processing enzymes, such as mannosylphosphate transferase, may also have to be eliminated depending on the host's particular endogenous glycosylation pattern.

Sugar Nucleotide Precursors

[0009] The *N*-glycans of animal glycoproteins typically include galactose, fucose, and terminal sialic acid. These sugars are not found on glycoproteins produced in yeast and filamentous fungi. In humans, the full range of nucleotide sugar precursors (e.g. UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, CMP-*N*-acetylneuraminic acid, UDP-galactose, GDP-fucose, etc.) are synthesized in the cytosol and transported into the Golgi, where they are attached to the core oligosaccharide by glycosyltransferases. (Sommers and Hirschberg, 1981 J. Cell Biol. 91(2): A406-A406; Sommers and Hirschberg 1982 *J. Biol. Chem.* 257(18): 811-817; Perez and Hirschberg 1987 *Methods in Enzymology* 138: 709-715).

[0010] Glycosyl transfer reactions typically yield a side product which is a nucleoside diphosphate or monophosphate. While monophosphates can be directly exported in exchange for nucleoside triphosphate sugars by an antiport mechanism, diphosphonucleosides (e.g. GDP) have to be cleaved by phosphatases (e.g. GDPase) to yield nucleoside monophosphates and inorganic phosphate prior to being exported. This reaction is important for efficient glycosylation; for example, GDPase from *Saccharomyces cerevisiae (S.cerevisiae)* has been found to be

necessary for mannosylation. However that GDPase has 90% reduced activity toward UDP (Berninsone et al., 1994 *J. Biol. Chem.* 269(1):207-211). Lower eukaryotes typically lack UDP-specific diphosphatase activity in the Golgi since they do not utilize UDP-sugar precursors for Golgi-based glycoprotein synthesis. *Schizosaccharomyces pombe*, a yeast found to add galactose residues to cell wall polysaccharides (from UDP-galactose) has been found to have specific UDPase activity, indicating the potential requirement for such an enzyme (Berninsone et al., 1994). UDP is known to be a potent inhibitor of glycosyltransferases and the removal of this glycosylation side product may be important to prevent glycosyltransferase inhibition in the lumen of the Golgi (Khatara et al., 1974). See Berninsone, P., et al. 1995. *J. Biol. Chem.* 270(24): 14564-14567; Beaudet, L., et al. 1998 *Abc Transporters: Biochemical, Cellular, and Molecular Aspects*. 292: 397-413.

Sequential Processing of N-glycans by Compartmentalized Enzyme Activities

[0011] Sugar transferases and glycosidases (e.g., mannosidases) line the inner (luminal) surface of the ER and Golgi apparatus and thereby provide a "catalytic" surface that allows for the sequential processing of glycoproteins as they proceed through the ER and Golgi network. The multiple compartments of the cis, medial, and trans Golgi and the trans-Golgi Network (TGN), provide the different localities in which the ordered sequence of glycosylation reactions can take place. As a glycoprotein proceeds from synthesis in the ER to full maturation in the late Golgi or TGN, it is sequentially exposed to different glycosidases, mannosidases and glycosyltransferases such that a specific carbohydrate structure may be synthesized. Much work has been dedicated to revealing the exact mechanism by which these enzymes are retained and anchored to their respective organelle. The evolving picture is complex but evidence suggests that stem region, membrane spanning region and cytoplasmic tail, individually or in concert, direct enzymes to the membrane of individual organelles and thereby localize the associated catalytic domain to that locus (see, e.g., Gleeson, P.A. (1998) *Histochem. Cell Biol.* 109, 517-532).

[0012] In some cases, these specific interactions were found to function across species. For example, the membrane spanning domain of α2,6-ST from rats, an enzyme known to localize in the trans-Golgi of the animal, was shown to also localize a reporter gene (invertase) in the yeast Golgi (Schwientek, 1995). However, the very same membrane spanning domain as part of a full-length α2,6-ST was retained in the ER and not further transported to the Golgi of yeast (Krezdorn, 1994). A full length GalT from humans was not even synthesized in yeast, despite demonstrably high transcription levels. In contrast, the transmembrane region of the same human GalT fused to an invertase reporter was able to direct localization to the yeast Golgi, albeit it at low production levels. Schwientek and co-workers have shown that fusing 28 amino acids of a yeast mannosyltransferase (*MNT1*), a region containing a cytoplasmic tail, a transmembrane region and eight amino acids of the stem region, to the catalytic domain of human GalT are sufficient for Golgi localization of an active GalT. Other galactosyltransferases appear to rely on interactions with enzymes resident in particular organelles because, after removal of their transmembrane region, they are still able to localize properly.

[0013] Improper localization of a glycosylation enzyme may prevent proper functioning of the enzyme in the pathway. For example, *Aspergillus nidulans*, which has numerous α-1,2-mannosidases (Eades and Hintz, 2000 *Gene* 255(1):25-34), does not add GlcNAc to $Man_5GlcNAc_2$ when transformed with the rabbit GnTI gene, despite a high overall level of GnTI activity (Kalsner et al., 1995). GnTI, although actively expressed, may be incorrectly localized such that the enzyme is not in contact with both of its substrates: UDP-GlcNAc and a productive $Man_5GlcNAc_2$ substrate (not all $Man_5GlcNAc_2$ structures are productive; see below). Alternatively, the host organism may not provide an adequate level of UDP-GlcNAc in the Golgi or the enzyme may be properly localized but nevertheless inactive in its new environment. In addition, $Man_5GlcNAc_2$ structures present in the host cell may differ in structure from $Man_5GlcNAc_2$ found in mammals. Maras and coworkers found that about one third of the N-glycans from cellobiohydrolase I (CBHI) obtained from *T.reesei* can be trimmed to $Man_5GlcNAc_2$ by *A.saitoi* 1,2 mannosidase *in vitro*. Fewer than 1%

of those N-glycans, however, could serve as a productive substrate for GnTI. The mere presence of $Man_5GlcNAc_2$, therefore, does not assure that further processing to $Man_5GlcNAc_2$ can be achieved. It is formation of a productive, GnTI-reactive $Man_5GlcNAc_2$ structure that is required. Although $Man_5GlcNAc_2$ could be produced in the cell (about 27 mol %), only a small fraction could be converted to $Man_5GlcNAc_2$ (less than about 5%, see Chiba WO 01/14522).

[0014] To date, there is no reliable way of predicting whether a particular heterologously expressed glycosyltransferase or mannosidase in a lower eukaryote will be (1), sufficiently translated (2), catalytically active or (3) located to the proper organelle within the secretory pathway. Because all three of these are necessary to affect glycosylation patterns in lower eukaryotes, a systematic scheme to achieve the desired catalytic function and proper retention of enzymes in the absence of predictive tools, which are currently not available, would be desirable.

Production of Therapeutic Glycoproteins

[0015] A significant number of proteins isolated from humans or animals are post-translationally modified, with glycosylation being one of the most significant modifications. An estimated 70% of all therapeutic proteins are glycosylated and thus currently rely on a production system (i.e., host cell) that is able to glycosylate in a manner similar to humans. Several studies have shown that glycosylation plays an important role in determining the (1) immunogenicity, (2) pharmacokinetic properties, (3) trafficking, and (4) efficacy of therapeutic proteins. It is thus not surprising that substantial efforts by the pharmaceutical industry have been directed at developing processes to obtain glycoproteins that are as "humanoid" or "human-like" as possible. To date, most glycoproteins are made in a mammalian host system. This may involve the genetic engineering of such mammalian cells to enhance the degree of sialylation (i.e., terminal addition of sialic acid) of proteins expressed by the cells, which is known to improve pharmacokinetic properties of such proteins. Alternatively, one may improve the degree of sialylation by *in vitro* addition of such sugars using known glycosyltransferases and their respective nucleotide sugars (e.g., 2,3-sialyltransferase and CMP-sialic acid).

[0016] While most higher eukaryotes carry out glycosylation reactions that are similar to those found in humans, recombinant human proteins expressed in the above mentioned host systems invariably differ from their "natural" human counterpart (Raju, 2000). Extensive development work has thus been directed at finding ways to improve the "human character" of proteins made in these expression systems. This includes the optimization of fermentation conditions and the genetic modification of protein expression hosts by introducing genes encoding enzymes involved in the formation of human-like glycoforms (Werner, 1998; Weikert, 1999; Andersen, 1994; Yang, 2000). Inherent problems associated with all mammalian expression systems have not been solved.

[0017] Fermentation processes based on mammalian cell culture (e.g., CHO, murine, or human cells), for example, tend to be very slow (fermentation times in excess of one week are not uncommon), often yield low product titers, require expensive nutrients and cofactors (e.g., bovine fetal serum), are limited by programmed cell death (apoptosis), and often do not enable expression of particular therapeutically valuable proteins. More importantly, mammalian cells are susceptible to viruses that have the potential to be human pathogens and stringent quality controls are required to assure product safety. This is of particular concern as many such processes require the addition of complex and temperature sensitive media components that are derived from animals (e.g., bovine calf serum), which may carry agents pathogenic to humans such as bovine spongiform encephalopathy (BSE) prions or viruses. Moreover, the production of therapeutic compounds is preferably carried out in a well-controlled sterile environment. An animal farm, no matter how cleanly kept, does not constitute such an environment, thus constituting an additional problem in the use of transgenic animals for manufacturing high volume therapeutic proteins.

[0018] Most, if not all, currently produced therapeutic glycoproteins are therefore expressed in mammalian cells and much effort has been directed at improving (i.e., "humanizing") the glycosylation pattern of these recombinant proteins. Changes in medium composition as well as the co-expression of genes encoding enzymes involved in human glycosylation have been successfully employed (see, for example, Weikert, 1999).

Glycoprotein Production Using Eukaryotic Microorganisms

[0019] The lack of a suitable mammalian expression system is a significant obstacle to the low-cost and safe production of recombinant human glycoproteins for therapeutic applications. It would be desirable to produce recombinant proteins similar to their mammalian, e.g., human, counterparts in lower eukaryotes (fungi and yeast). Production of glycoproteins via the fermentation of microorganisms would offer numerous advantages over existing systems. For example, fermentation-based processes may offer (a) rapid production of high concentrations of protein; (b) the ability to use sterile, well-controlled production conditions; (c) the ability to use simple, chemically defined (and protein-free) growth media; (d) ease of genetic manipulation; (e) the absence of contaminating human or animal pathogens such as viruses; (f) the ability to express a wide variety of proteins, including those poorly expressed in cell culture owing to toxicity etc.; and (g) ease of protein recovery (e.g. via secretion into the medium). In addition, fermentation facilities for yeast and fungi are generally far less costly to construct than cell culture facilities. Although the core oligosaccharide structure transferred to a protein in the endoplasmic reticulum is basically identical in mammals and lower eukaryotes, substantial differences have been found in the subsequent processing reactions which occur in the Golgi apparatus of fungi and mammals. In fact, even amongst different lower eukaryotes there exist a great variety of glycosylation structures. This has historically prevented the use of lower eukaryotes as hosts for the production of recombinant human glycoproteins despite otherwise notable advantages over mammalian expression systems.

[0020] Therapeutic glycoproteins produced in a microorganism host such as yeast utilizing the endogenous host glycosylation pathway differ structurally from those produced in mammalian cells and typically show greatly reduced therapeutic efficacy. Such glycoproteins are typically immunogenic in humans and show a reduced half-life (and thus bioactivity) *in vivo* after administration (Takeuchi, 1997). Specific receptors in humans and animals (i.e., macrophage mannose receptors) can recognize terminal mannose residues and promote the rapid clearance of the foreign glycoprotein from the bloodstream. Additional adverse

effects may include changes in protein folding, solubility, susceptibility to proteases, trafficking, transport, compartmentalization, secretion, recognition by other proteins or factors, antigenicity, or allergenicity.

[0021] Yeast and filamentous fungi have both been successfully used for the production of recombinant proteins, both intracellular and secreted (Cereghino, J. L. and J. M. Cregg 2000 *FEMS Microbiology Reviews* 24(1): 45-66; Harkki, A., et al. 1989 *Bio-Technology* 7(6): 596; Berka, R. M., et al. 1992 *Abstr.Papers Amer. Chem.Soc.*203: 121-BIOT; Svetina, M., et al. 2000 *J. Biotechnol.* 76(2-3): 245-251). Various yeasts, such as *K. lactis*, *Pichia pastoris, Pichia methanolica,* and *Hansenula polymorpha,* have played particularly important roles as eukaryotic expression systems because they are able to grow to high cell densities and secrete large quantities of recombinant protein. Likewise, filamentous fungi, such as *Aspergillus niger, Fusarium sp, Neurospora crassa* and others, have been used to efficiently produce glycoproteins at the industrial scale. However, as noted above, glycoproteins expressed in any of these eukaryotic microorganisms differ substantially in *N*-glycan structure from those in animals. This has prevented the use of yeast or filamentous fungi as hosts for the production of many therapeutic glycoproteins.

[0022] Although glycosylation in yeast and fungi is very different than in humans, some common elements are shared. The first step, the transfer of the core oligosaccharide structure to the nascent protein, is highly conserved in all eukaryotes including yeast, fungi, plants and humans (compare **Figs. 1A** and **1B**). Subsequent processing of the core oligosaccharide, however, differs significantly in yeast and involves the addition of several mannose sugars. This step is catalyzed by mannosyltransferases residing in the Golgi (e.g. *OCH1*, *MNT1*, *MNN1*, etc.), which sequentially add mannose sugars to the core oligosaccharide. The resulting structure is undesirable for the production of humanoid proteins and it is thus desirable to reduce or eliminate mannosyltransferase activity. Mutants of *S.cerevisiae* deficient in mannosyltransferase activity (e.g. *och1* or *mnn9* mutants) have shown to be non-lethal and display a reduced mannose content in the oligosaccharide of yeast glycoproteins. Other oligosaccharide processing enzymes, such as mannosylphosphate transferase, may also have to be eliminated

depending on the host's particular endogenous glycosylation pattern. After reducing undesired endogenous glycosylation reactions, the formation of complex N-glycans has to be engineered into the host system. This requires the stable expression of several enzymes and sugar-nucleotide transporters. Moreover, one has to localize these enzymes so that a sequential processing of the maturing glycosylation structure is ensured.

[0023] Several efforts have been made to modify the glycosylation pathways of eukaryotic microorganisms to provide glycoproteins more suitable for use as mammalian therapeutic agents. For example, several glycosyltransferases have been separately cloned and expressed in *S cerevisiae* (GalT, GnTI), *Aspergillus nidulans* (GnTI) and other fungi (Yoshida et al., 1999, Kalsner et al., 1995 *Glycoconj. J.* 12(3):360-370, Schwientek et al., 1995). However, *N*-glycans resembling those made in human cells were not obtained.

[0024] Yeasts produce a variety of mannosyltransferases (e.g., 1,3-mannosyltransferases such as *MNN1* in *S. cerevisiae;* Graham and Emr, 1991 *J. Cell. Biol.* 114(2):207-218), 1,2-mannosyltransferases (e.g. *KTR/KRE* family from S.*cerevisiae*), 1,6-mannosyltransferases (e.g., *OCH1* from *S.cerevisiae*), mannosylphosphate transferases and their regulators (*e.g.*, *MNN4* and *MNN6* from *S.cerevisiae*) and additional enzymes that are involved in endogenous glycosylation reactions. Many of these genes have been deleted individually giving rise to viable organisms having altered glycosylation profiles. Examples are shown in **Table 1.**

Table 1. Examples of yeast strains having altered mannosylation

Strain	*N*-glycan (wild type)	Mutation	*N*-glycan (mutant)	Reference
S. pombe	$Man_{>9}GlcNAc_2$	OCH1	$Man_8GlcNAc_2$	Yoko-o et al., 2001 *FEBS Lett.* 489(1):75-80
S.cerevisiae	$Man_{>9}GlcNAc_2$	OCH1/MNN1	$Man_8GlcNAc_2$	Nakanishi-Shindo et al,. 1993 *J. Biol. Chem.* 268(35):26338-26345
S.cerevisiae	$Man_{>9}GlcNAc_2$	OCH1/MNN1/MNN4	$Man_8GlcNAc_2$	Chiba et al., 1998 *J. Biol. Chem.* 273, 26298-26304
P.pastoris	Hyperglycosylated	OCH1 (complete deletion)	Not hyperglycosylated	Welfide, Japanese Application Publication No. 8-

P.pastoris	$Man_{>8}GlcNAc_2$	OCH1 (disruption)	$Man_{>8}GlcNAc_2$	336387 Contreras et al. WO 02/00856 A2

[0025] Japanese Patent Application Publication No. 8-336387 discloses the deletion of an *OCH1* homolog in *Pichia pastoris*. In *S.cerevisiae, OCH1* encodes a 1,6-mannosyltransferase, which adds a mannose to the glycan structure $Man_8GlcNAc_2$ to yield $Man_9GlcNAc_2$. The $Man_9GlcNAc_2$ structure, which contains three 1,6 mannose residues, is then a substrate for further 1,2-, 1,6-, and 1,3- mannosyltransferases *in vivo*, leading to the hypermannosylated glycoproteins that are characteristic for *S.cerevisiae* and which typically may have 30-40 mannose residues per *N*-glycan. Because the Och1p initiates the transfer of 1,6 mannose to the $Man_8GlcNAc_2$ core, it is often referred to as the "initiating 1,6 mannosyltransferase" to distinguish it from other 1,6 mannosyltransferases acting later in the Golgi. In an *och1 mnn1 mnn4* mutant strain of *S.cerevisiae*, proteins glycosylated with $Man_8GlcNAc_2$ accumulate and hypermannosylation does not occur. However, $Man_8GlcNAc_2$ is not a substrate for mammalian glycosyltransferases, such as human UDP-GlcNAc transferase I, and accordingly, the use of that mutant strain, in itself, is not useful for producing mammalian-like proteins, i.e., with complex or hybrid glycosylation patterns.

[0026] Although Japanese Patent Application Publication No. 8-336387 discloses methods to obtain an *och1* mutant of *P.pastoris* displaying a reduced mannosylation phenotype, it provides no data on whether the initiating 1,6 mannosyltransferase activity presumed to be encoded by *OCH1* is reduced or eliminated. It is well-established in the field of fungal genetics that homologs of genes often do not play the same role in their respective host organism. For example, the *Neurospora rca-1* gene complements an *Aspergillus flbD* sporulation mutant but has no identifiable role in *Neurospora* sporulation. Shen, W.C. et al., *Genetics* 1998;148(3):1031-41. More recently, Contreras (WO 02/00856 A2) shows that, in an *och1* mutant of *P. pastoris*, at least 50% of the cell wall glycans cannot be trimmed to $Man_5GlcNAc_2$ with a *Trichoderma reesei* α-1,2-mannosidase (see Fig. 11 of WO 02/00856 A2). As the wild-type displays a very similar glycosylation pattern (Fig. 10, Panel 2 of WO 02/00856 A2), it appears that the *OCH1* gene of *P.pastoris* may not encode the initiating 1,6-mannosyltransferase

activity and is thus different from its genetic homolog in *S.cerevisiae*. Thus, to date, there is no evidence that initiating α-1,6-mannosyltransferase activity is eliminated in *och1* mutants of *P. pastoris*, which further supports the notion that the glycosylation pathways of *S.cerevisiae* and *P.pastoris* are significantly different.

[0027] Martinet et al. (*Biotechnol. Lett.* 1998, 20(12), 1171-1177) reported the expression of α-1,2-mannosidase from *T. reesei* in *P. pastoris*. Some mannose trimming from the *N*-glycans of a model protein was observed. However, the model protein had no *N*-glycans with the structure $Man_5GlcNAc_2$, which would be necessary as an intermediate for the generation of complex *N*-glycans. Accordingly, that system is not useful for producing proteins with complex or hybrid glycosylation patterns.

[0028] Similarly, Chiba et al. (1998) expressed α-1,2-mannosidase from *Aspergillus saitoi* in the yeast *Saccharomyces cerevisiae*. A signal peptide sequence (His-Asp-Glu-Leu) **(SEQ ID NO: 5)** was engineered into the exogenous mannosidase to promote its retention in the endoplasmic reticulum. In addition, the yeast host was a mutant lacking enzyme activities associated with hypermannosylation of proteins: 1,6-mannosyltransferase (*och1*); 1,3-mannosyltransferase (*mnn1*); and a regulator of mannosylphosphate transferase (*mnn4*). The *N*-glycans of the triple mutant host consisted primarily of the structure $Man_8GlcNAc_2$, rather than the high mannose forms found in wild-type *S.cerevisiae*. In the presence of the engineered mannosidase, the *N*-glycans of a model protein (carboxypeptidase Y) were trimmed to give a mixture consisting of 27 mole % $Man_5GlcNAc_2$, 22 mole % $Man_6GlcNAc_2$, 22 mole % $Man_7GlcNAc_2$, and 29 mole % $Man_8GlcNAc_2$. Trimming of cell wall glycoproteins was less efficient, with only 10 mole % of the *N*-glycans having the desired $Man_5GlcNAc_2$ structure.

[0029] Even if all the $Man_5GlcNAc_2$ glycans were the correct $Man_5GlcNAc_2$ form that can be converted to $GlcNAcMan_5GlcNAc_2$ by GnTI, the above system would not be efficient for the production of proteins having human-like glycosylation patterns. If several glycosylation sites are present in a desired protein, the probability (P) of obtaining such a protein in a correct form follows the

relationship $P=(F)^n$, where n equals the number of glycosylation sites, and F equals the fraction of desired glycoforms. A glycoprotein with three glycosylation sites would have a 0.1% chance of providing the appropriate precursors for complex and hybrid N-glycan processing on all of its glycosylation sites. Thus, using the system of Chiba to make a glycoprotein having a single *N*-glycosylation site, at least 73 mole % would have an incorrect structure. For a glycoprotein having two or three *N*-glycosylation sites, at least 93 or 98 mole % would have an incorrect structure, respectively. Such low efficiencies of conversion are unsatisfactory for the production of therapeutic agents, particularly as the separation of proteins having different glycoforms is typically costly and difficult.

[0030] Chiba et al. (WO 01/14522) have shown high levels of $Man_5GlcNAc_2$ structures on recombinant fibroblast growth factor (FGF), a secreted soluble glycoprotein produced in *S.cerevisiae*. It is not clear, however, that the detected $Man_5GlcNAc_2$ was produced inside the host cell (i.e. *in vivo*) because the α-1,2 mannosidase was targeted by fusion with an HDEL **(SEQ ID NO: 5)** localization tag, a mechanism, which is known to be leaky (Pelham H.R. (1998) *EMBO J.* 7, 913-918). It is more likely that FGF was secreted into the medium, where it was then processed by α-1,2 mannosidase which had escaped the HDEL **(SEQ ID NO: 5)** retrieval mechanism and leaked into the medium. As mentioned above, an intracellular protein (CPY), expressed in the same strain, contained mostly glycans (more than 73%) that were $Man_6GlcNAc_2$ and larger. The majority of the $Man_5GlcNAc_2$ structures on FGF are, thus, likely to have been produced *ex vivo*. It is further unclear whether the $Man_5GlcNAc_2$ structures that were produced were productive substrates for GnTI.

[0031] As the above work demonstrates, one can trim $Man_8GlcNAc_2$ structures to a $Man_5GlcNAc_2$ isomer in *S.cerevisiae,* although high efficiency trimming greater than 50% *in vivo* has yet to be determined, by engineering a fungal mannosidase from *A. saitoi* into the endoplasmic reticulum (ER). The shortcomings of this approach are two-fold: (1) it is not clear whether the $Man_5GlcNAc_2$ structures formed are in fact formed *in vivo* (rather than having been secreted and further modified by mannosidases outside the cell); and (2) it is not clear whether any $Man_5GlcNAc_2$ structures formed, if in fact formed *in vivo*,

are the correct isoform to be a productive substrate for subsequent *N*-glycan modification by GlcNAc transferase I (Maras et al., 1997, *Eur. J. Biochem.* 249, 701-707).

[0032] With the objective of providing a more human-like glycoprotein derived from a fungal host, U.S. Patent No. 5,834,251 discloses a method for producing a hybrid glycoprotein derived from *Trichoderma reseei*. A hybrid *N*-glycan has only mannose residues on the Manα1-6 arm of the core mannose structure and one or two complex antennae on the Manα1-3 arm. While this structure has utility, the method has the disadvantage that numerous enzymatic steps must be performed *in vitro*, which is costly and time-consuming. Isolated enzymes are expensive to prepare and need costly substrates (e.g. UDP-GlcNAc). The method also does not allow for the production of complex glycans on a desired protein.

Intracellular Mannosidase Activity Involved in N-glycan Trimming

[0033] Alpha-1,2-mannosidase activity is required for the trimming of $Man_8GlcNAc_2$ to form $Man_5GlcNAc_2$, which is a major intermediate for complex *N*-glycan formation in mammals. Previous work has shown that truncated murine, fungal and human α-1,2-mannosidase can be expressed in the methylotropic yeast *P.pastoris* and display $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ trimming activity (Lal et al., *Glycobiology* 1998 Oct;8(10):981-95; Tremblay et al., *Glycobiology* 1998 Jun;8(6):585-95, Callewaert et al., 2001). However, to date, no reports exist that show the high level *in vivo* trimming of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ on a secreted glycoprotein from *P.pastoris*.

[0034] While it is useful to engineer strains that are able to produce $Man_5GlcNAc_2$ as the primary *N*-glycan structure, any attempt to further modify these high mannose precursor structures to more closely resemble human glycans requires additional *in vivo* or *in vitro* steps. Methods to further humanize glycans from fungal and yeast sources *in vitro* are described in U.S. Pat. No. 5,834,251 (*supra*). As discussed above, however, if $Man_5GlcNAc_2$ is to be further humanized *in vivo*, one has to ensure that the generated $Man_5GlcNAc_2$ structures are, in fact, generated intracellularly and not the product of mannosidase activity in the medium. Complex *N*-glycan formation in yeast or fungi will require high levels of

$Man_5GlcNAc_2$ to be generated within the cell because only intracellular $Man_5GlcNAc_2$ glycans can be further processed to hybrid and complex *N*-glycans *in vivo*. In addition, one has to demonstrate that the majority of $Man_5GlcNAc_2$ structures generated are in fact a substrate for GnTI and thus allow the formation of hybrid and complex N-glycans.

[0035] Moreover, the mere presence of an α-1,2-mannosidase in the cell does not, by itself, ensure proper intracellular trimming of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$. (See, e.g., Contreras et al. WO 02/00856 A2, in which an HDEL **(SEQ ID NO: 5)** tagged mannosidase of *T. reesei* is localized primarily in the ER and co-expressed with an influenza haemagglutinin (HA) reporter protein on which virtually no $Man_5GlcNAc_2$ could be detected. See also Chiba et al., 1998 (*supra*), in which a chimeric α-1,2-mannosidase/Och1p transmembrane domain fusion localized in the ER, early Golgi and cytosol of *S.cerevisiae*, had no mannosidase trimming activity). Accordingly, mere localization of a mannosidase in the ER or Golgi is insufficient to ensure activity of the respective enzyme in that targeted organelle. (See also, Martinet et al. (1998), *supra*, showing that α-1,2-mannosidase from *T. reesei*, while localizing intracellularly, increased rather than decreased the extent of mannosylation). To date, there is no report that demonstrates the intracellular localization of an active heterologous α-1,2-mannosidase in either yeast or fungi using a transmembrane localization sequence.

[0036] Accordingly, the need exists for methods to produce glycoproteins characterized by a high intracellular $Man_5GlcNAc_2$ content which can be further processed into human-like glycoprotein structures in non-human eukaryotic host cells, and particularly in yeast and filamentous fungi.

SUMMARY OF THE INVENTION

[0037] Host cells and cell lines having genetically modified glycosylation pathways that allow them to carry out a sequence of enzymatic reactions which mimic the processing of glycoproteins in mammals, especially in humans, have been developed. Recombinant proteins expressed in these engineered hosts yield glycoproteins more similar, if not substantially identical, to their mammalian, e.g., human counterparts. Host cells of the invention, e.g., lower eukaryotic micro-

organisms and other non-human, eukaryotic host cells grown in culture, are modified to produce *N*-glycans such as $Man_5GlcNAc_2$ or other structures produced along human glycosylation pathways. This is achieved using a combination of engineering and/or selection of strains which: do not express certain enzymes which create the undesirable structures characteristic of the fungal glycoproteins; which express heterologous enzymes selected either to have optimal activity under the conditions present in the host cell where activity is to be achieved; or combinations thereof; wherein the genetically engineered eukaryote expresses at least one heterologous enzyme activity required to produce a "human-like" glycoprotein. Host cells of the invention may be modified further by heterologous expression of one or more activities such as glycosyltransferases, sugar transporters and mannosidases, to become strains for the production of mammalian, e.g., human therapeutic glycoproteins.

[0038] The present invention thus provides a glycoprotein production method using (1) a lower eukaryotic host such as a unicellular or filamentous fungus, or (2) any non-human eukaryotic organism that has a different glycosylation pattern from humans, to modify the glycosylation composition and structures of the proteins made in a host organism ("host cell") so that they resemble more closely carbohydrate structures found in mammalian, e.g., human proteins. The process allows one to obtain an engineered host cell which can be used to express and target any desirable gene(s), e.g.,one involved in glycosylation, by methods that are well-established in the scientific literature and generally known to the artisan in the field of protein expression. Host cells with modified oligosaccharides are created or selected. N-glycans made in the engineered host cells have a $Man_5GlcNAc_2$ core structure which may then be modified further by heterologous expression of one or more enzymes, e.g., glycosyltransferases, glycosidases, sugar transporters and mannosidases, to yield human-like glycoproteins. For the production of therapeutic proteins, this method may be adapted to engineer cell lines in which any desired glycosylation structure may be obtained.

[0039] Accordingly, in one embodiment, the invention provides a method for producing a human-like glycoprotein in a non-human eukaryotic host cell. The host cell of the invention is selected or engineered to be depleted in 1,6-mannosyl-

transferase activities which would otherwise add mannose residues onto the N-glycan on a glycoprotein. One or more enzymes (enzymatic activities) are introduced into the host cell which enable the production of a $Man_5GlcNAc_2$ carbohydrate structure at a high yield, e.g., at least 30 mole percent. In a more preferred embodiment, at least 10% of the $Man_5GlcNAc_2$ produced within the host cell is a productive substrate for GnTI and thus for further glycosylation reactions *in vivo* and/or *in vitro* that produce a finished *N*-glycan that is similar or identical to that formed in mammals, especially humans.

[0040] In another embodiment, a nucleic acid molecule encoding one or more enzymes for production of a $Man_5GlcNAc_2$ carbohydrate structure is introduced into a host cell selected or engineered to be depleted in 1,6-mannosyltransferase activities. In one preferred embodiment, at least one enzyme introduced into the host cell is selected to have optimal activity at the pH of the subcellular location where the carbohydrate structure is produced. In another preferred embodiment, at least one enzyme is targeted to a host subcellular organelle where the enzyme will have optimal activity, e.g., by means of a chimeric protein comprising a cellular targeting signal peptide not normally associated with the enzyme.

[0041] The invention further provides isolated nucleic acid molecules and vectors comprising such molecules which encode an initiating α1,6-mannosyl-transferase activity isolated from *P.pastoris* or from *K.lactis*. These nucleic acid molecules comprise sequences that are homologous to the *OCH1* gene in *S.cerevisiae*. These and homologous sequences are useful for constructing host cells which will not hypermannosylate the N-glycan of a glycoprotein.

[0042] In another embodiment, the host cell is engineered to express a heterologous glycosidase, e.g., by introducing into the host one or more nucleic acid molecules encoding the glycosidase. Preferably, a nucleic acid molecule encodes one or more mannosidase activities involved in the production of $Man_5GlcNAc_2$ from $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$. In a preferred embodiment, at least one of the encoded mannosidase activities has a pH optimum within 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the mannosidase activity is localized, or has optimal activity at a pH of between about 5.1 and about 8.0, preferably between about 5.5 and about 7.5.

Preferably, the heterologous enzyme is targeted to the endoplasmic reticulum, the Golgi apparatus or the transport vesicles between ER and Golgi of the host organism, where it trims *N*-glycans such as $Man_8GlcNAc_2$ to yield high levels of $Man_5GlcNAc_2$. In one embodiment, the enzyme is targeted by forming a fusion protein between a catalytic domain of the enzyme and a cellular targeting signal peptide, e.g., by the in-frame ligation of a DNA fragment encoding a cellular targeting signal peptide with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof.

[0043] In yet another embodiment, the glycosylation pathway of a host is modified to express a sugar nucleotide transporter. In a preferred embodiment, a nucleotide diphosphatase enzyme is also expressed. The transporter and diphosphatase improve the efficiency of engineered glycosylation steps, by providing the appropriate substrates for the glycosylation enzymes in the appropriate compartments, reducing competitive product inhibition, and promoting the removal of nucleoside diphosphates.

[0044] The present invention also provides a combinatorial nucleic acid library useful for making fusion constructs which can target a desired protein or polypeptide fragment, e.g., an enzyme involved in glycosylation or a catalytic domain thereof, to a selected subcellular region of a host cell. In one preferred embodiment, the combinatorial nucleic acid library comprises (a) nucleic acid sequences encoding different cellular targeting signal peptides and (b) nucleic acid sequences encoding different polypeptides to be targeted. Nucleic acid sequences of or derived from (a) and (b) are ligated together to produce fusion constructs, at least one of which encodes a functional protein domain (e.g., a catalytic domain of an enzyme) ligated in-frame to a heterologous cellular targeting signal peptide, i.e., one which it normally does not associate with.

[0045] The invention also provides a method for modifying the glycosylation pathway of a host cell (e.g., any eukaryotic host cell, including a human host cell) using enzymes involved in modifying N-glycans including glycosidases and glycosyltransferases; by transforming the host cell with a nucleic acid (e.g., a combinatorial) library of the invention to produce a genetically mixed cell population expressing at least one and preferably two or more distinct chimeric

glycosylation enzymes having a catalytic domain ligated in-frame to a cellular targeting signal peptide which it normally does not associate with. A host cell having a desired glycosylation phenotype may optionally be selected from the population. Host cells modified using the library and associated methods of the invention are useful, e.g., for producing glycoproteins having a glycosylation pattern similar or identical to those produced in mammals, especially humans.

[0046] In another aspect, the combinatorial library of the present invention enables production of one or a combination of catalytically active glycosylation enzymes, which successfully localize to intracellular compartments in which they function efficiently in the glycosylation/secretory pathway. Preferred enzymes convert $Man_5(\alpha\text{-}1,2\text{-}Man)_{3\text{-}9}GlcNAc_2$ to $Man_5GlcNAc_2$ at high efficiency *in vivo*. In addition, the invention provides eukaryotic host strains, and in particular, yeasts, fungal, insect, plant, plant cells, algae and insect cell hosts, capable of producing glycoprotein intermediates or products with $Man_5GlcNAc_2$ and/or $GlcNAcMan_5GlcNAc_2$ as the predominant *N*-glycan.

[0047] The present invention also provides recombinant molecules derived from a combinatorial nucleic acid library; vectors, including expression vectors, comprising such recombinant molecules; proteins encoded by the recombinant molecules and vectors; host cells transformed with the recombinant molecules or vectors; glycoproteins produced from such transformed hosts; and methods for producing, *in vivo*, glycoprotein intermdiates or products with predominantly $Man_5GlcNAc_2$ or $GlcNAcMan_5GlcNAc_2$ *N*-glycans covalently attached to appropriate glycosylation sites using the combinatorial library.

[0048] Further aspects of this invention include methods, compositions and kits for diagnostic and therapeutic uses in which presence or absence of $Man_5GlcNAc_2$ and/or $GlcNAcMan_5GlcNAc_2$ on a glycoprotein may be detected.

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] **Figure 1A** is a schematic diagram of a typical fungal *N*-glycosylation pathway.

[0050] **Figure 1B** is a schematic diagram of a typical human *N*-glycosylation pathway.

[0051] **Figure 2** depicts construction of a combinatorial DNA library of fusion constructs. **Fig. 2A** diagrams the insertion of a targeting peptide fragment into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). **Fig. 2B** shows the generated targeting peptide sub-library having restriction sites *NotI* - *AscI*. **Fig. 2C** diagrams the insertion of a catalytic domain region into pJN347, a modified pUC19 vector. **Fig. 2D** shows the generated catalytic domain sub-library having restriction sites *NotI*, *AscI* and *PacI*. **Fig. 2E** depicts one particular fusion construct generated from the targeting peptide sub-library and the catalytic domain sub-library.

[0052] **Figure 3 (SEQ ID NOS: 45-46** respectively, in order of appearance) illustrates the *M.musculus* α-1,2-mannosidase IA open reading frame. The sequences of the PCR primers used to generate N-terminal truncations are underlined.

[0053] **Figures 4A – 4F** illustrates engineering of vectors with multiple auxotrophic markers and genetic integration of target proteins in the *P.pastoris* *OCH1* locus.

[0054] **Figures 5A – 5E** show MALDI-TOF analysis demonstrating production of kringle 3 domain of human plasminogen (K3) glycoproteins having $Man_5GlcNAc_2$ as the predominant *N*-glycan structure in *P. pastoris*. **Fig. 5A** depicts the standard $Man_5GlcNAc_2$ [**a**] glycan (Glyko, Novato, CA) and $Man_5GlcNAc_2 + Na^+$ [**b**]. **Fig. 5B** shows PNGase – released glycans from K3 wild type. The *N*-glycans shown are as follows: $Man_9GlcNAc_2$ [**d**]; $Man_{10}GlcNAc_2$ [**e**]; $Man_{11}GlcNAc_2$ [**f**]; $Man_{12}GlcNAc_2$ [**g**]. **Fig. 5C** depicts the *och1* deletion resulting in the production of $Man_8GlcNAc_2$ [**c**] as the predominant *N*-glycan. **Figs. 5D** and **5E** show the production of $Man_5GlcNAc_2$ [**b**] after *in vivo* trimming of $Man_8GlcNAc_2$ with a chimeric α-1,2-mannosidase. The predominant *N*-glycan is indicated by a peak with a mass (m/z) of 1253 consistent with its identification as $Man_5GlcNAc_2$ [**b**].

[0055] **Figures 6A – 6F** show MALDI-TOF analysis demonstrating production of IFN-β glycoproteins having $Man_5GlcNAc_2$ as the predominant *N*-glycan structure in *P. pastoris*. **Fig. 6A** shows the standard $Man_5GlcNAc_2$ [**a**] and $Man_5GlcNAc_2 + Na^+$ [**b**] as the standard (Glyko, Novato, CA). **Fig. 6B** shows PNGase – released glycans from IFN- β wildtype. **Fig. 6C** depicts the *och1* knock-

out producing $Man_8GlcNAc_2$ [**c**]; $Man_9GlcNAc_2$ [**d**]; $Man_{10}GlcNAc_2$ [**e**]; $Man_{11}GlcNAc_2$ [**f**]; $Man_{12}GlcNAc_2$ [**g**]; and no production of $Man_5GlcNAc_2$ [**b**]. **Fig. 6D** shows relatively small amount of $Man_5GlcNAc_2$ [**b**] among other intermediate *N*-glycans $Man_8GlcNAc_2$ [**c**] to $Man_{12}GlcNAc_2$ [**g**]. **Fig. 6E** shows a significant amount of $Man_5GlcNAc_2$ [**b**] relative to the other glycans $Man_8GlcNAc_2$ [**c**] and $Man_9GlcNAc_2$ [**d**] produced by pGC5 (*Saccharomyces MNS1*(m)/mouse mannosidase IB Δ99). **Fig. 6F** shows predominant production of $Man_5GlcNAc_2$ [**b**] on the secreted glycoprotein IFN-β by pFB8 (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187). The *N*-glycan is indicated by a peak with a mass (m/z) of 1254 consistent with its identification as $Man_5GlcNAc_2$ [**b**].

[0056] **Figure 7** shows a high performance liquid chromatogram for: (**A**) $Man_9GlcNAc_2$ standard labeled with 2-AB (negative control); (**B**) supernatant of medium *P.pastoris*, *Δoch1* transformed with pFB8 mannosidase, which demonstrates a lack of extracellular mannosidase activity in the supernatant; and (**C**) $Man_9GlcNAc_2$ standard labeled with 2-AB after exposure to *T.reesei* mannosidase (positive control).

[0057] **Figure 8** shows a high performance liquid chromatogram for: (**A**) $Man_9GlcNAc_2$ standard labeled with 2-AB (negative control); (**B**) supernatant of medium *P.pastoris*, *Δoch1* transformed with pGC5 mannosidase, which demonstrates a lack of extracellular mannosidase activity in the supernatant; and (**C**) $Man_9GlcNAc_2$ standard labeled with 2-AB after exposure to *T.reesei* mannosidase (positive control).

[0058] **Figure 9** shows a high performance liquid chromatogram for: (**A**) $Man_9GlcNAc_2$ standard labeled with 2-AB (negative control); (**B**) supernatant of medium *P.pastoris*, *Δoch1* transformed with pBC18-5 mannosidase, which demonstrates lack of extracellular mannosidase activity in the supernatant; and (**C**) supernatant of medium *P.pastoris*, *Δoch1* transformed with pDD28-3, which demonstrates activity in the supernatant (positive control).

[0059] **Figures 10A – 10B** demonstrate the activity of an UDP-GlcNAc transporter in the production of $GlcNAcMan_5GlcNAc_2$ in *P. pastoris*. **Fig. 10A** depicts a *P.pastoris* strain (YSH-3) with a human GnTI but without the UDP-GlcNAc transporter resulting in some production of $GlcNAcMan_5GlcNAc_2$ [**b**] but

a predominant production of $Man_5GlcNAc_2$ [**a**]. **Fig 10B** depicts the addition of UDP-GlcNAc transporter from *K.lactis* in a strain (PBP-3) with the human GnTI, which resulted in the predominant production of $GlcNAcMan_5GlcNAc_2$ [**b**]. The single prominent peak of mass (m/z) at 1457 is consistent with its identification as $GlcNAcMan_5GlcNAc_2$ [**b**] as shown in **Fig. 10B**.

[0060] **Figure 11** shows a pH optimum of a heterologous mannosidase enzyme encoded by pBB27-2 (*Saccharomyces MNN10* (s)/*C. elegans* mannosidase IB Δ31) expressed in *P. pastoris*.

[0061] **Figures 12A – 12C** show MALDI-TOF analysis of *N*-glycans released from a cell free extract of *K.lactis*. **Fig. 12A** shows the *N*-glycans released from wild-type cells, which includes high-mannose type *N*-glycans. **Fig. 12B** shows the *N*-glycans released from *och1 mnn1* deleted cells, revealing a distinct peak of mass (m/z) at 1908 consistent with its identification as $Man_9GlcNAc_2$ [**d**]. **Fig. 12C** shows the *N*-glycans released from *och1 mnn1* deleted cells after *in vitro* α-1,2-mannosidase digest corresponding to a peak consistent with $Man_5GlcNAc_2$.

[0062] **Figure 13** represents T-DNA cassettes with catalytic domain(s) of glycosylation enzymes fused in-frame to different leader sequences. The ends of the T-DNA are marked by the right (rb) and left borders (lb). Various promoters and terminators may also be used. The plant selectable marker can also be varied. The right and left borders are required only for agrobacterium-mediated transformation and not for particle bombardment or electroporation.

DETAILED DESCRIPTION OF THE INVENTION

[0063] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein

and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art.

[0064] The methods and techniques of the present invention are generally performed according to conventional methods well-known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992, and Supplements to 2002); Harlow and Lane Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990); Introduction to Glycobiology, Maureen E. Taylor, Kurt Drickamer, Oxford Univ. Press (2003); Worthington Enzyme Manual, Worthington Biochemical Corp. Freehold, NJ; Handbook of Biochemistry: Section A Proteins Vol I 1976 CRC Press; Handbook of Biochemistry: Section A Proteins Vol II 1976 CRC Press; Essentials of Glycobiology, Cold Spring Harbor Laboratory Press (1999). The nomenclatures used in connection with, and the laboratory procedures and techniques of, molecular and cellular biology, protein biochemistry, enzymology and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art.

[0065] All publications, patents and other references mentioned herein are incorporated by reference.

[0066] The following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0067] As used herein, the term "N-glycan" refers to an N-linked oligosaccharide, e.g., one that is attached by an asparagine-N-acetylglucosamine linkage to an asparagine residue of a polypeptide. N-glycans have a common pentasaccharide core of $Man_3GlcNAc_2$ ("Man" refers to mannose; "Glc" refers to glucose; and "NAc" refers to N-acetyl; GlcNAc refers to N-acetylglucosamine). The term " trimannose core" used with respect to the N-glycan also refers to the structure $Man_3GlcNAc_2$ ("Man_3"). N-glycans differ with respect to the number of branches (antennae) comprising peripheral sugars (e.g., fucose and sialic acid) that

are added to the Man_3 core structure. N-glycans are classified according to their branched constituents (e.g., high mannose, complex or hybrid).

[0068] A "high mannose" type N-glycan has five or more mannose residues. A "complex" type N-glycan typically has at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of the trimannose core. Complex N-glycans may also have galactose ("Gal") residues that are optionally modified with sialic acid or derivatives ("NeuAc", where "Neu" refers to neuraminic acid and "Ac" refers to acetyl). A complex N-glycan typically has at least one branch that terminates in an oligosaccharide such as, for example: NeuNAc-; NeuAca2-6GalNAca1-; NeuAca2-3Galb1-3GalNAca1-; NeuAca2-3/6Galb1-4GlcNAcb1-; GlcNAca1-4Galb1-(mucins only); Fuca1-2Galb1-(blood group H). Sulfate esters can occur on galactose, GalNAc, and GlcNAc residues, and phosphate esters can occur on mannose residues. NeuAc (Neu: neuraminic acid; Ac:acetyl) can be O-acetylated or replaced by NeuGl (N-glycolylneuraminic acid). Complex N-glycans may also have intrachain substitutions comprising "bisecting" GlcNAc and core fucose ("Fuc"). A "hybrid" N-glycan has at least one GlcNAc on the terminal of the 1,3 mannose arm of the trimannose core and zero or more mannoses on the 1,6 mannose arm of the trimannose core.

[0069] The term "predominant" or "predominantly" used with respect to the production of N-glycans refers to a structure which represents the major peak detected by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) analysis.

[0070] Abbreviations used herein are of common usage in the art, see, e.g., abbreviations of sugars, above. Other common abbreviations include "PNGase", which refers to peptide N-glycosidase F (EC 3.2.2.18); "GlcNAc Tr" or "GnT," which refers to *N*-acetylglucosaminyl Transferase enzymes; "NANA" refers to *N*-acetylneuraminic acid.

[0071] As used herein, a "humanized glycoprotein" or a "human-like glycoprotein" refers alternatively to a protein having attached thereto N-glycans having less than four mannose residues, and synthetic glycoprotein intermediates (which are also useful and can be manipulated further *in vitro* or *in vivo*) having at least five mannose residues. Preferably, glycoproteins produced according to the

invention contain at least 30 mole %, preferably at least 40 mole % and more preferably 50 – 100 mole % of the $Man_5GlcNAc_2$ intermediate, at least transiently. This may be achieved, e.g., by engineering a host cell of the invention to express a "better", i.e., a more efficient glycosylation enzyme. For example, a mannosidase is selected such that it will have optimal activity under the conditions present at the site in the host cell where proteins are glycosylated and is introduced into the host cell preferably by targeting the enzyme to a host cell organelle where activity is desired.

[0072] The term "enzyme", when used herein in connection with altering host cell glycosylation, refers to a molecule having at least one enzymatic activity, and includes full-length enzymes, catalytically active fragments, chimerics, complexes, and the like. A "catalytically active fragment" of an enzyme refers to a polypeptide having a detectable level of functional (enzymatic) activity.

[0073] A lower eukaryotic host cell, when used herein in connection with glycosylation profiles, refers to any eukaryotic cell which ordinarily produces high mannose containing N-glycans, and thus is meant to include some animal or plant cells and most typical lower eukaryotic cells, including uni- and multicellular fungal and algal cells.

[0074] As used herein, the term "secretion pathway" refers to the assembly line of various glycosylation enzymes to which a lipid-linked oligosaccharide precursor and an N-glycan substrate are sequentially exposed, following the molecular flow of a nascent polypeptide chain from the cytoplasm to the endoplasmic reticulum (ER) and the compartments of the Golgi apparatus. Enzymes are said to be localized along this pathway. An enzyme X that acts on a lipid-linked glycan or an N-glycan before enzyme Y is said to be or to act "upstream" to enzyme Y; similarly, enzyme Y is or acts "downstream" from enzyme X.

[0075] The term "targeting peptide" as used herein refers to nucleotide or amino acid sequences encoding a cellular targeting signal peptide which mediates the localization (or retention) of an associated sequence to sub-cellular locations, e.g., organelles.

[0076] The term "polynucleotide" or "nucleic acid molecule" refers to a polymeric form of nucleotides of at least 10 bases in length. The term includes

DNA molecules (e.g., cDNA or genomic or synthetic DNA) and RNA molecules (e.g., mRNA or synthetic RNA), as well as analogs of DNA or RNA containing non-natural nucleotide analogs, non-native internucleoside bonds, or both. The nucleic acid can be in any topological conformation. For instance, the nucleic acid can be single-stranded, double-stranded, triple-stranded, quadruplexed, partially double-stranded, branched, hairpinned, circular, or in a padlocked conformation. The term includes single and double stranded forms of DNA. A nucleic acid molecule of this invention may include both sense and antisense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. They may be modified chemically or biochemically or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, etc.), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids, etc.) Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

[0077] Unless otherwise indicated, a "nucleic acid comprising SEQ ID NO:X" refers to a nucleic acid, at least a portion of which has either (i) the sequence of SEQ ID NO:X, or (ii) a sequence complementary to SEQ ID NO:X. The choice between the two is dictated by the context. For instance, if the nucleic acid is used as a probe, the choice between the two is dictated by the requirement that the probe be complementary to the desired target.

[0078] An "isolated" or "substantially pure" nucleic acid or polynucleotide (e.g., an RNA, DNA or a mixed polymer) is one which is substantially separated from other cellular components that naturally accompany the native polynucleotide in its natural host cell, e.g., ribosomes, polymerases, and genomic sequences with which

it is naturally associated. The term embraces a nucleic acid or polynucleotide that (1) has been removed from its naturally occurring environment, (2) is not associated with all or a portion of a polynucleotide in which the "isolated polynucleotide" is found in nature, (3) is operatively linked to a polynucleotide which it is not linked to in nature, or (4) does not occur in nature. The term "isolated" or "substantially pure" also can be used in reference to recombinant or cloned DNA isolates, chemically synthesized polynucleotide analogs, or polynucleotide analogs that are biologically synthesized by heterologous systems.

[0079] However, "isolated" does not necessarily require that the nucleic acid or polynucleotide so described has itself been physically removed from its native environment. For instance, an endogenous nucleic acid sequence in the genome of an organism is deemed "isolated" herein if a heterologous sequence (i.e., a sequence that is not naturally adjacent to this endogenous nucleic acid sequence) is placed adjacent to the endogenous nucleic acid sequence, such that the expression of this endogenous nucleic acid sequence is altered. By way of example, a non-native promoter sequence can be substituted (e.g., by homologous recombination) for the native promoter of a gene in the genome of a human cell, such that this gene has an altered expression pattern. This gene would now become "isolated" because it is separated from at least some of the sequences that naturally flank it.

[0080] A nucleic acid is also considered "isolated" if it contains any modifications that do not naturally occur to the corresponding nucleic acid in a genome. For instance, an endogenous coding sequence is considered "isolated" if it contains an insertion, deletion or a point mutation introduced artificially, e.g., by human intervention. An "isolated nucleic acid" also includes a nucleic acid integrated into a host cell chromosome at a heterologous site, a nucleic acid construct present as an episome. Moreover, an "isolated nucleic acid" can be substantially free of other cellular material, or substantially free of culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

[0081] As used herein, the phrase "degenerate variant" of a reference nucleic acid sequence encompasses nucleic acid sequences that can be translated,

according to the standard genetic code, to provide an amino acid sequence identical to that translated from the reference nucleic acid sequence.

[0082] The term "percent sequence identity" or "identical" in the context of nucleic acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over a stretch of at least about nine nucleotides, usually at least about 20 nucleotides, more usually at least about 24 nucleotides, typically at least about 28 nucleotides, more typically at least about 32 nucleotides, and preferably at least about 36 or more nucleotides. There are a number of different algorithms known in the art which can be used to measure nucleotide sequence identity. For instance, polynucleotide sequences can be compared using FASTA, Gap or Bestfit, which are programs in Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisconsin. FASTA provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson, 1990, herein incorporated by reference). For instance, percent sequence identity between nucleic acid sequences can be determined using FASTA with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) or using Gap with its default parameters as provided in GCG Version 6.1, herein incorporated by reference.

[0083] The term "substantial homology" or "substantial similarity," when referring to a nucleic acid or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 50%, more preferably 60% of the nucleotide bases, usually at least about 70%, more usually at least about 80%, preferably at least about 90%, and more preferably at least about 95%, 96%, 97%, 98% or 99% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or Gap, as discussed above.

[0084] Alternatively, substantial homology or similarity exists when a nucleic acid or fragment thereof hybridizes to another nucleic acid, to a strand of another nucleic acid, or to the complementary strand thereof, under stringent hybridization conditions. "Stringent hybridization conditions" and "stringent wash conditions"

in the context of nucleic acid hybridization experiments depend upon a number of different physical parameters. Nucleic acid hybridization will be affected by such conditions as salt concentration, temperature, solvents, the base composition of the hybridizing species, length of the complementary regions, and the number of nucleotide base mismatches between the hybridizing nucleic acids, as will be readily appreciated by those skilled in the art. One having ordinary skill in the art knows how to vary these parameters to achieve a particular stringency of hybridization.

[0085] In general, "stringent hybridization" is performed at about 25°C below the thermal melting point (T_m) for the specific DNA hybrid under a particular set of conditions. "Stringent washing" is performed at temperatures about 5°C lower than the T_m for the specific DNA hybrid under a particular set of conditions. The T_m is the temperature at which 50% of the target sequence hybridizes to a perfectly matched probe. See Sambrook et al., *supra*, page 9.51, hereby incorporated by reference. For purposes herein, "high stringency conditions" are defined for solution phase hybridization as aqueous hybridization (i.e., free of formamide) in 6X SSC (where 20X SSC contains 3.0 M NaCl and 0.3 M sodium citrate), 1% SDS at 65°C for 8-12 hours, followed by two washes in 0.2X SSC, 0.1% SDS at 65°C for 20 minutes. It will be appreciated by the skilled artisan that hybridization at 65°C will occur at different rates depending on a number of factors including the length and percent identity of the sequences which are hybridizing.

[0086] The term "mutated" when applied to nucleic acid sequences means that nucleotides in a nucleic acid sequence may be inserted, deleted or changed compared to a reference nucleic acid sequence. A single alteration may be made at a locus (a point mutation) or multiple nucleotides may be inserted, deleted or changed at a single locus. In addition, one or more alterations may be made at any number of loci within a nucleic acid sequence. A nucleic acid sequence may be mutated by any method known in the art including but not limited to mutagenesis techniques such as "error-prone PCR" (a process for performing PCR under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product. See, e.g., Leung, D. W., et al., *Technique*, 1, pp. 11-15 (1989) and Caldwell, R. C.

& Joyce G. F., *PCR Methods Applic.*, 2, pp. 28-33 (1992)); and "oligonucleotide-directed mutagenesis" (a process which enables the generation of site-specific mutations in any cloned DNA segment of interest. See, e.g., Reidhaar-Olson, J. F. & Sauer, R. T., et al., *Science*, 241, pp. 53-57 (1988)).

[0087] The term "vector" as used herein is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Other vectors include cosmids, bacterial artificial chromosomes (BAC) and yeast artificial chromosomes (YAC). Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome (discussed in more detail below). Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., vectors having an origin of replication which functions in the host cell). Other vectors can be integrated into the genome of a host cell upon introduction into the host cell, and are thereby replicated along with the host genome. Moreover, certain preferred vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors").

[0088] "Operatively linked" expression control sequences refers to a linkage in which the expression control sequence is contiguous with the gene of interest to control the gene of interest, as well as expression control sequences that act in *trans* or at a distance to control the gene of interest.

[0089] The term "expression control sequence" as used herein refers to polynucleotide sequences which are necessary to affect the expression of coding sequences to which they are operatively linked. Expression control sequences are sequences which control the transcription, post-transcriptional events and translation of nucleic acid sequences. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (e.g., ribosome binding sites); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such

control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is essential for expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0090] The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a nucleic acid such as a recombinant vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. A recombinant host cell may be an isolated cell or cell line grown in culture or may be a cell which resides in a living tissue or organism.

[0091] The term "peptide" as used herein refers to a short polypeptide, e.g., one that is typically less than about 50 amino acids long and more typically less than about 30 amino acids long. The term as used herein encompasses analogs and mimetics that mimic structural and thus biological function.

[0092] The term "polypeptide" as used herein encompasses both naturally-occurring and non-naturally-occurring proteins, and fragments, mutants, derivatives and analogs thereof. A polypeptide may be monomeric or polymeric. Further, a polypeptide may comprise a number of different domains each of which has one or more distinct activities.

[0093] The term "isolated protein" or "isolated polypeptide" is a protein or polypeptide that by virtue of its origin or source of derivation (1) is not associated with naturally associated components that accompany it in its native state, (2) when it exists in a purity not found in nature, where purity can be adjudged with respect to the presence of other cellular material (e.g., is free of other proteins from the same species) (3) is expressed by a cell from a different species, or (4) does not occur in nature (e.g., it is a fragment of a polypeptide found in nature or it includes amino acid analogs or derivatives not found in nature or linkages other than

standard peptide bonds). Thus, a polypeptide that is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally originates will be "isolated" from its naturally associated components. A polypeptide or protein may also be rendered substantially free of naturally associated components by isolation, using protein purification techniques well-known in the art. As thus defined, "isolated" does not necessarily require that the protein, polypeptide, peptide or oligopeptide so described has been physically removed from its native environment.

[0094] The term "polypeptide fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion compared to a full-length polypeptide. In a preferred embodiment, the polypeptide fragment is a contiguous sequence in which the amino acid sequence of the fragment is identical to the corresponding positions in the naturally-occurring sequence. Fragments typically are at least 5, 6, 7, 8, 9 or 10 amino acids long, preferably at least 12, 14, 16 or 18 amino acids long, more preferably at least 20 amino acids long, more preferably at least 25, 30, 35, 40 or 45, amino acids, even more preferably at least 50 or 60 amino acids long, and even more preferably at least 70 amino acids long.

[0095] A "modified derivative" refers to polypeptides or fragments thereof that are substantially homologous in primary structural sequence but which include, e.g., *in vivo* or *in vitro* chemical and biochemical modifications or which incorporate amino acids that are not found in the native polypeptide. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labeling, e.g., with radionuclides, and various enzymatic modifications, as will be readily appreciated by those well skilled in the art. A variety of methods for labeling polypeptides and of substituents or labels useful for such purposes are well-known in the art, and include radioactive isotopes such as ^{125}I, ^{32}P, ^{35}S, and ^{3}H, ligands which bind to labeled antiligands (e.g., antibodies), fluorophores, chemiluminescent agents, enzymes, and antiligands which can serve as specific binding pair members for a labeled ligand. The choice of label depends on the sensitivity required, ease of conjugation with the primer, stability requirements, and available instrumentation. Methods for labeling

polypeptides are well-known in the art. See Ausubel et al., 1992, hereby incorporated by reference.

[0096] A "polypeptide mutant" or "mutein" refers to a polypeptide whose sequence contains an insertion, duplication, deletion, rearrangement or substitution of one or more amino acids compared to the amino acid sequence of a native or wild type protein. A mutein may have one or more amino acid point substitutions, in which a single amino acid at a position has been changed to another amino acid, one or more insertions and/or deletions, in which one or more amino acids are inserted or deleted, respectively, in the sequence of the naturally-occurring protein, and/or truncations of the amino acid sequence at either or both the amino or carboxy termini. A mutein may have the same but preferably has a different biological activity compared to the naturally-occurring protein. For instance, a mutein may have an increased or decreased neuron or NgR binding activity. In a preferred embodiment of the present invention, a MAG derivative that is a mutein (e.g., in MAG Ig-like domain 5) has decreased neuronal growth inhibitory activity compared to endogenous or soluble wild-type MAG.

[0097] A mutein has at least 70% overall sequence homology to its wild-type counterpart. Even more preferred are muteins having 80%, 85% or 90% overall sequence homology to the wild-type protein. In an even more preferred embodiment, a mutein exhibits 95% sequence identity, even more preferably 97%, even more preferably 98% and even more preferably 99% overall sequence identity. Sequence homology may be measured by any common sequence analysis algorithm, such as Gap or Bestfit.

[0098] Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinity or enzymatic activity, and (5) confer or modify other physicochemical or functional properties of such analogs.

[0099] As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. *See Immunology - A Synthesis* (2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Mass. (1991)), which is incorporated herein by reference. Stereoisomers (e.g., D-amino

acids) of the twenty conventional amino acids, unnatural amino acids such as α-, α-disubstituted amino acids, N-alkyl amino acids, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, s-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right hand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

[0100] A protein has "homology" or is "homologous" to a second protein if the nucleic acid sequence that encodes the protein has a similar sequence to the nucleic acid sequence that encodes the second protein. Alternatively, a protein has homology to a second protein if the two proteins have "similar" amino acid sequences. (Thus, the term "homologous proteins" is defined to mean that the two proteins have similar amino acid sequences). In a preferred embodiment, a homologous protein is one that exhibits 60% sequence homology to the wild type protein, more preferred is 70% sequence homology. Even more preferred are homologous proteins that exhibit 80%, 85% or 90% sequence homology to the wild type protein. In a yet more preferred embodiment, a homologous protein exhibits 95%, 97%, 98% or 99% sequence identity. As used herein, homology between two regions of amino acid sequence (especially with respect to predicted structural similarities) is interpreted as implying similarity in function.

[0101] When "homologous" is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of homology may be adjusted upwards to correct for

the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art (see, e.g., Pearson et al., 1994, herein incorporated by reference).

[0102] The following six groups each contain amino acids that are conservative substitutions for one another: 1) Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Alanine (A), Valine (V), and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[0103] Sequence homology for polypeptides, which is also referred to as percent sequence identity, is typically measured using sequence analysis software. See, e.g., the Sequence Analysis Software Package of the Genetics Computer Group (GCG), University of Wisconsin Biotechnology Center, 910 University Avenue, Madison, Wisconsin 53705. Protein analysis software matches similar sequences using measure of homology assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG contains programs such as "Gap" and "Bestfit" which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutein thereof. See, e.g., GCG Version 6.1.

[0104] A preferred algorithm when comparing a inhibitory molecule sequence to a database containing a large number of sequences from different organisms is the computer program BLAST (Altschul, S.F. et al. (1990) *J. Mol. Biol.* 215:403-410; Gish and States (1993) *Nature Genet.* 3:266-272; Madden, T.L. et al. (1996) *Meth. Enzymol.* 266:131-141; Altschul, S.F. et al. (1997) *Nucleic Acids Res.*25:3389-3402; Zhang, J. and Madden, T.L. (1997) *Genome Res.* 7:649-656), especially blastp or tblastn (Altschul et al., 1997). Preferred parameters for BLASTp are: Expectation value: 10 (default); Filter: seg (default); Cost to open a gap: 11 (default); Cost to extend a gap: 1 (default); Max. alignments: 100 (default); Word size: 11 (default); No. of descriptions: 100 (default); Penalty Matrix: BLOWSUM62.

[0105] The length of polypeptide sequences compared for homology will generally be at least about 16 amino acid residues, usually at least about 20 residues, more usually at least about 24 residues, typically at least about 28 residues, and preferably more than about 35 residues. When searching a database containing sequences from a large number of different organisms, it is preferable to compare amino acid sequences. Database searching using amino acid sequences can be measured by algorithms other than blastp known in the art. For instance, polypeptide sequences can be compared using FASTA, a program in GCG Version 6.1. FASTA provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson, 1990, herein incorporated by reference). For example, percent sequence identity between amino acid sequences can be determined using FASTA with its default parameters (a word size of 2 and the PAM250 scoring matrix), as provided in GCG Version 6.1, herein incorporated by reference.

[0106] The term "fusion protein" refers to a polypeptide comprising a polypeptide or fragment coupled to heterologous amino acid sequences. Fusion proteins are useful because they can be constructed to contain two or more desired functional elements from two or more different proteins. A fusion protein comprises at least 10 contiguous amino acids from a polypeptide of interest, more preferably at least 20 or 30 amino acids, even more preferably at least 40, 50 or 60 amino acids, yet more preferably at least 75, 100 or 125 amino acids. Fusion proteins can be produced recombinantly by constructing a nucleic acid sequence which encodes the polypeptide or a fragment thereof in-frame with a nucleic acid sequence encoding a different protein or peptide and then expressing the fusion protein. Alternatively, a fusion protein can be produced chemically by crosslinking the polypeptide or a fragment thereof to another protein.

[0107] The term "region" as used herein refers to a physically contiguous portion of the primary structure of a biomolecule. In the case of proteins, a region is defined by a contiguous portion of the amino acid sequence of that protein.

[0108] The term "domain" as used herein refers to a structure of a biomolecule that contributes to a known or suspected function of the biomolecule. Domains may be co-extensive with regions or portions thereof; domains may also include

distinct, non-contiguous regions of a biomolecule. Examples of protein domains include, but are not limited to, an Ig domain, an extracellular domain, a transmembrane domain, and a cytoplasmic domain.

[0109] As used herein, the term "molecule" means any compound, including, but not limited to, a small molecule, peptide, protein, sugar, nucleotide, nucleic acid, lipid, etc., and such a compound can be natural or synthetic.

[0110] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice of the present invention and will be apparent to those of skill in the art. All publications and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

[0111] Throughout this specification and claims, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Methods For Producing Host Cells Having $Man_5GlcNAc_2$ Modified Oligosaccharides For The Generation of Human-like N-Glycans

[0112] The invention provides a method for producing a glycoprotein having human-like glycosylation in a non-human eukaryotic host cell. As described in more detail below, a eukaryotic host cell that does not naturally express, or which is engineered not to express, one or more enzymes involved in production of high mannose structures is selected as a starting host cell. Such a selected host cell is engineered to express one or more enzymes or other factors required to produce human-like glycoproteins. A desired host strain can be engineered one enzyme or more than one enzyme at a time. In addition, a nucleic acid molecule encoding one or more enzymes or activities may be used to engineer a host strain of the invention. Preferably, a library of nucleic acid molecules encoding potentially

useful enzymes (e.g., chimeric enzymes comprising a catalytically active enzyme fragment ligated in-frame to a heterologous subcellular targeting sequence) is created (e.g., by ligation of sub-libraries comprising enzymatic fragments and subcellular targeting sequences), and a strain having one or more enzymes with optimal activities or producing the most "human-like" glycoproteins may be selected by transforming target host cells with one or more members of the library.

[0113] In particular, the methods described herein enable one to obtain, *in vivo*, $Man_5GlcNAc_2$ structures in high yield, at least transiently, for the purpose of further modifying it to yield complex N-glycans. A successful scheme to obtain suitable $Man_5GlcNAc_2$ structures in appropriate yields in a host cell, such as a lower eukaryotic organism, generally involves two parallel approaches: (1) reducing high mannose structures made by endogenous mannosyltransferase activities, if any, and (2) removing 1,2- α- mannose by mannosidases to yield high levels of suitable $Man_5GlcNAc_2$ structures which may be further reacted inside the host cell to form complex, human-like glycoforms.

[0114] Accordingly, a first step involves the selection or creation of a eukaryotic host cell, e.g., a lower eukaryote, capable of producing a specific precursor structure of $Man_5GlcNAc_2$ that is able to accept *in vivo* GlcNAc by the action of a GlcNAc transferase I ("GnTI"). In one embodiment, the method involves making or using a non-human eukaryotic host cell depleted in a 1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein. Preferably, the host cell is depleted in an initiating 1,6 mannosyltransferase activity (see below). Such a host cell will lack one or more enzymes involved in the production of high mannose structures which are undesirable for producing human-like glycoproteins.

[0115] One or more enzyme activities are then introduced into such a host cell to produce N-glycans within the host cell characterized by having at least 30 mol % of the $Man_5GlcNAc_2$ ("Man_5") carbohydrate structures. $Man_5GlcNAc_2$ structures are necessary for complex N-glycan formation: $Man_5GlcNAc_2$ must be formed *in vivo* in a high yield (e.g., in excess of 30%), at least transiently, as subsequent mammalian- and human-like glycosylation reactions require $Man_5GlcNAc_2$ or a derivative thereof.

[0116] This step also requires the formation of a particular isomeric structure of $Man_5GlcNAc_2$ within the cell at a high yield. While $Man_5GlcNAc_2$ structures are necessary for complex N-glycan formation, their presence is by no means sufficient. That is because $Man_5GlcNAc_2$ may occur in different isomeric forms, which may or may not serve as a substrate for GlcNAc transferase I. As most glycosylation reactions are not complete, a particular glycosylated protein generally contains a range of different carbohydrate structures (i.e. glycoforms) on its surface. Thus, the mere presence of trace amounts (i.e., less than 5%) of a particular structure like $Man_5GlcNAc_2$ is of little practical relevance for producing mammalian- or human-like glycoproteins. It is the formation of a GlcNAc transferase I-accepting $Man_5GlcNAc_2$ intermediate (**Fig. 1B**) in high yield (i.e., above 30%), which is required. The formation of this intermediate is necessary to enable subsequent *in vivo* synthesis of complex N-glycans on glycosylated proteins of interest (target proteins).

[0117] Accordingly, some or all of the $Man_5GlcNAc_2$ produced by the selected host cell must be a productive substrate for enzyme activities along a mammalian glycosylation pathway, e.g., can serve as a substrate for a GlcNAc transferase I activity *in vivo*, thereby forming the human-like N-glycan intermediate $GlcNAcMan_5GlcNAc_2$ in the host cell. In a preferred embodiment, at least 10%, more preferably at least 30% and most preferably 50% or more of the $Man_5GlcNAc_2$ intermediate produced in the host cell of the invention is a productive substrate for GnTI *in vivo*. It is understood that if, for example, $GlcNAcMan_5GlcNAc_2$ is produced at 10% and $Man_5GlcNAc_2$ is produced at 25% on a target protein, that the total amount of transiently produced $Man_5GlcNAc_2$ is 35% because $GlcNAcMan_5GlcNAc_2$ is a product of $Man_5GlcNAc_2$.

[0118] One of ordinary skill in the art can select host cells from nature, e.g., existing fungi or other lower eukaryotes that produce significant levels of $Man_5GlcNAc_2$ *in vivo*. As yet, however, no lower eukaryote has been shown to provide such structures *in vivo* in excess of 1.8% of the total N-glycans (see e.g. Maras et al., 1997). Alternatively, such host cells may be genetically engineered to produce the $Man_5GlcNAc_2$ structure *in vivo*. Methods such as those described in U.S. Patent No. 5,595,900 may be used to identify the absence or presence of

particular glycosyltransferases, mannosidases and sugar nucleotide transporters in a target host cell or organism of interest.

Inactivation of Undesirable Host Cell Glycosylation Enzymes

[0119] The methods of the invention are directed to making host cells which produce glycoproteins having altered, and preferably human-like, N-glycan structures. In a preferred embodiment, the methods are directed to making host cells in which oligosaccharide precursors are enriched in $Man_5GlcNAc_2$. Preferably, a eukaryotic host cell is used that does not express one or more enzymes involved in the production of high mannose structures. Such a host cell may be found in nature or may be engineered, e.g., starting with or derived from one of many such mutants already described in yeasts. Thus, depending on the selected host cell, one or a number of genes that encode enzymes known to be characteristic of non-human glycosylation reactions will have to be deleted. Such genes and their corresponding proteins have been extensively characterized in a number of lower eukaryotes (e.g., *S.cerevisiae, T. reesei, A. nidulans* etc.), thereby providing a list of known glycosyltransferases in lower eukaryotes, their activities and their respective genetic sequence. These genes are likely to be selected from the group of mannosyltransferases e.g. 1,3 mannosyltransferases (e.g. *MNN1* in *S.cerevisiae*) (Graham, 1991), 1,2 mannosyltransferases (e.g. *KTR/KRE* family from *S.cerevisiae*), 1,6 mannosyltransferases (*OCH1* from *S.cerevisiae*), mannosylphosphate transferases and their regulators (*MNN4* and *MNN6* from *S.cerevisiae*) and additional enzymes that are involved in aberrant, i.e. non human, glycosylation reactions. Many of these genes have in fact been deleted individually giving rise to viable phenotypes with altered glycosylation profiles. Examples are shown in **Table 1.**

[0120] Preferred lower eukaryotic host cells of the invention, as described herein to exemplify the required manipulation steps, are hypermannosylation-minus (*och1*) mutants of *Pichia pastoris* or *K.lactis*. Like other lower eukaryotes, *P.pastoris* processes $Man_9GlcNAc_2$ structures in the ER with an α-1,2-mannosidase to yield $Man_8GlcNAc_2$ (**Fig. 1A**). Through the action of several mannosyltransferases, this structure is then converted to hypermannosylated

structures ($Man_{>9}GlcNAc_2$), also known as mannans. In addition, it has been found that *P.pastoris* is able to add non-terminal phosphate groups, through the action of mannosylphosphate transferases, to the carbohydrate structure. This differs from the reactions performed in mammalian cells, which involve the removal rather than addition of mannose sugars. It is of particular importance to eliminate the ability of the eukaryotic host cell, e.g., fungus, to hypermannosylate an existing $Man_8GlcNAc_2$ structure. This can be achieved by either selecting for a host cell that does not hypermannosylate or by genetically engineering such a cell.

[0121] Genes that are involved in the hypermannosylation process have been identified, e.g., in *Pichia pastoris*, and by creating mutations in these genes, one can reduce the production of "undesirable" glycoforms. Such genes can be identified by homology to existing mannosyltransferases or their regulators (e.g., *OCH1*, *MNN4*, *MNN6*, *MNN1*) found in other lower eukaryotes such as *C. albicans*, *Pichia angusta* or *S.cerevisiae* or by mutagenizing the host strain and selecting for a glycosylation phenotype with reduced mannosylation. Based on homologies amongst known mannosyltransferases and mannosylphosphate transferases, one may either design PCR primers (**SEQ ID NOS: 7, 8, 47** and 4 left to right, respectively, in order of appearance) (examples of which are shown in **Table 2**), or use genes or gene fragments encoding such enzymes as probes to identify homologs in DNA libraries of the target or a related organism. Alternatively, one may identify a functional homolog having mannosyltransferase activity by its ability to complement particular glycosylation phenotypes in related organisms.

Table 2. PCR Primers

PCR primer A	PCR primer B	Target Gene(s) in *P.pastoris*	Homologs
ATGGCGAAGGCAGA TGGCAGT	TTAGTCCTTCCAAC TTCCTTC	1,6-mannosyltransferase	OCH1 *S.cerevisiae*, *Pichia albicans*
TAYTGGMGNGTNGA RCYNGAYATHAA	GCRTCNCCCCANCK YTCRTA	1,2 mannosyltransferases	KTR/KRE family, *S.cerevisiae*

Legend: M = A or C, R = A or G, W = A or T, S = C or G, Y = C or T, K = G or T, V = A or C or G, H = A or C or T, D = A or G or T, B = C or G or T, N = G or A or T or C.

[0122] To obtain the gene or genes encoding 1,6-mannosyltransferase activity in *P. pastoris,* for example, one would carry out the following steps: *OCH1* mutants of *S.cerevisiae* are temperature sensitive and are slow growers at elevated temperatures. One can thus identify functional homologs of *OCH1* in *P.pastoris* by complementing an *OCH1* mutant of *S.cerevisiae* with a *P.pastoris* DNA or cDNA library. Mutants of *S.cerevisiae* are available, e.g., from Stanford University and are commercially available from ResGen, an Invitrogen Corp. (Carlsbad, CA). Mutants that display a normal growth phenotype at elevated temperature, after having been transformed with a *P.pastoris* DNA library, are likely to carry an *OCH1* homolog of *P. pastoris*. Such a library can be created by partially digesting chromosomal DNA of *P.pastoris* with a suitable restriction enzyme and, after inactivating the restriction enzyme, ligating the digested DNA into a suitable vector, which has been digested with a compatible restriction enzyme.

[0123] Suitable vectors include, e.g., pRS314, a low copy (CEN6/ARS4) plasmid based on pBluescript containing the Trp1 marker (Sikorski, R. S., and Hieter, P.,1989, *Genetics* 122, pg 19-27) and pFL44S, a high copy (2μ) plasmid based on a modified pUC19 containing the URA3 marker (Bonneaud, N., et al., 1991, *Yeast* 7, pg. 609-615). Such vectors are commonly used by academic researchers and similar vectors are available from a number of different vendors (e.g., Invitrogen (Carlsbad, CA); Pharmacia (Piscataway, NJ); New England Biolabs (Beverly, MA)). Further examples include pYES/GS, 2μ origin of replication based yeast expression plasmid from Invitrogen, or Yep24 cloning vehicle from New England Biolabs.

[0124] After ligation of the chromosomal DNA and the vector, one may transform the DNA library into a strain of *S .cerevisiae* with a specific mutation and select for the correction of the corresponding phenotype. After sub-cloning and sequencing the DNA fragment that is able to restore the wild-type phenotype, one may use this fragment to eliminate the activity of the gene product encoded by *OCH1* in *P.pastoris* using *in vivo* mutagenesis and/or recombination techniques well-known to those skilled in the art.

[0125] Alternatively, if the entire genomic sequence of a particular host cell, e.g., fungus, of interest is known, one may identify such genes simply by searching publicly available DNA databases, which are available from several sources, such as NCBI, Swissprot. For example, by searching a given genomic sequence or database with sequences from a known 1,6 mannosyltransferase gene (e.g., *OCH1* from *S.cerevisiae*), one can identify genes of high homology in such a host cell genome which may (but do not necessarily) encode proteins that have 1,6-mannosyltransferase activity. Nucleic acid sequence homology alone is not enough to prove, however, that one has identified and isolated a homolog encoding an enzyme having the same activity. To date, for example, no data exist to show that an *OCH1* deletion in *P.pastoris* eliminates the crucial initiating 1,6-mannosyltransferase activity. (Martinet et al. *Biotech. Letters* 20(12) (Dec. 1998): 1171-1177; Contreras et al. WO 02/00856 A2). Thus, no data prove that the *P.pastoris OCH1* gene homolog actually encodes that function. That demonstration is provided for the first time herein.

[0126] Homologs to several *S.cerevisiae* mannosyltransferases have been identified in *P.pastoris* using these approaches. Homologous genes often have similar functions to genes involved in the mannosylation of proteins in *S.cerevisiae* and thus their deletion may be used to manipulate the glycosylation pattern in *P.pastoris* or, by analogy, in any other host cell, e.g., fungus, plant, insect or animal cells, with similar glycosylation pathways.

[0127] The creation of gene knock-outs, once a given target gene sequence has been determined, is a well-established technique in the art and can be carried out by one of ordinary skill in the art (see, e.g., R. Rothstein, (1991) Methods in Enzymology, vol. 194, p. 281). The choice of a host organism may be influenced by the availability of good transformation and gene disruption techniques.

[0128] If several mannosyltransferases are to be knocked out, the method developed by Alani and Kleckner, (*Genetics* 116:541-545 (1987)), for example, enables the repeated use of a selectable marker, e.g., the *URA3* marker in yeast, to sequentially eliminate all undesirable endogenous mannosyltransferase activity. This technique has been refined by others but basically involves the use of two repeated DNA sequences, flanking a counter selectable marker. For example:

URA3 may be used as a marker to ensure the selection of a transformants that have integrated a construct. By flanking the *URA3* marker with direct repeats one may first select for transformants that have integrated the construct and have thus disrupted the target gene. After isolation of the transformants, and their characterization, one may counter select in a second round for those that are resistant to 5-fluoroorotic acid (5-FOA). Colonies that are able to survive on plates containing 5-FOA have lost the *URA3* marker again through a crossover event involving the repeats mentioned earlier. This approach thus allows for the repeated use of the same marker and facilitates the disruption of multiple genes without requiring additional markers. Similar techniques for sequential elimination of genes adapted for use in another eukaryotic host cells with other selectable and counter-selectable markers may also be used.

[0129] Eliminating specific mannosyltransferases, such as 1,6 mannosyltransferase (*OCH1*) or mannosylphosphate transferases (*MNN6*, or genes complementing *lbd* mutants) or regulators (*MNN4*) in *P.pastoris* enables one to create engineered strains of this organism which synthesize primarily $Man_8GlcNAc_2$ and which can be used to further modify the glycosylation pattern to resemble more complex glycoform structures, e.g., those produced in mammalian, e.g., human cells. A preferred embodiment of this method utilizes DNA sequences encoding biochemical glycosylation activities to eliminate similar or identical biochemical functions in *P. pastoris* to modify the glycosylation structure of glycoproteins produced in the genetically altered *P.pastoris* strain.

[0130] Methods used to engineer the glycosylation pathway in yeasts as exemplified herein can be used in filamentous fungi to produce a preferred substrate for subsequent modification. Strategies for modifying glycosylation pathways in *A.niger* and other filamentous fungi, for example, can be developed using protocols analogous to those described herein for engineering strains to produce human-like glycoproteins in yeast. Undesired gene activities involved in 1,2 mannosyltransferase activity, e.g., *KTR/KRE* homologs, are modified or eliminated. A filamentous fungus, such as *Aspergillus*, is a preferred host because it lacks the 1,6 mannosyltransferase activity and as such, one would not expect a hypermannosylating gene activity, e.g. *OCH1*, in this host. By contrast, other

desired activities (e.g., α-1,2-mannosidase, UDP-GlcNAc transporter, glycosyltransferase (GnT), galactosyltransferase (GalT) and sialyltransferase (ST)) involved in glycosylation are introduced into the host using the targeting methods of the invention.

Engineering or Selecting Hosts Having Diminished Initiating α-1,6 Mannosyltransferase Activity

[0131] In a preferred embodiment, the method of the invention involves making or using a host cell which is diminished or depleted in the activity of an initiating α-1,6-mannosyltransferase, i.e., an initiation specific enzyme that initiates outer chain mannosylation on the α-1,3 arm of the $Man_3GlcNAc_2$ core structure. In *S.cerevisiae*, this enzyme is encoded by the *OCH1* gene. Disruption of the *OCH1* gene in *S.cerevisiae* results in a phenotype in which N-linked sugars completely lack the poly-mannose outer chain. Previous approaches for obtaining mammalian-type glycosylation in fungal strains have required inactivation of *OCH1* (see, e.g., Chiba, 1998). Disruption of the initiating α-1,6-mannosyltransferase activity in a host cell of the invention may be optional, however (depending on the selected host cell), as the Och1p enzyme requires an intact $Man_8GlcNAc_2$ for efficient mannose outer chain initiation. Thus, host cells selected or produced according to this invention which accumulate oligosaccharides having seven or fewer mannose residues may produce hypoglycosylated N-glycans that will likely be poor substrates for Och1p (see, e.g., Nakayama, 1997).

[0132] The *OCH1* gene was cloned from *P.pastoris* (**Example 1**) and *K.lactis* (**Example 16**), as described. The nucleic acid and amino acid sequences of the *OCH1* gene from *K.lactis* are set forth in SEQ ID NOS: 41 and 42. Using gene-specific primers, a construct was made from each clone to delete the *OCH1* gene from the genome of *P.pastoris* and *K.lactis* (**Examples 1 and 16**, respectively). Host cells depleted in initiating α-1,6-mannosyltransferase activity and engineered to produce N-glycans having a $Man_5GlcNAc_2$ carbohydrate structure were thereby obtained (see, e.g., **Figs. 5** and **6; Examples 11 and 16**).

[0133] Thus, in another embodiment, the invention provides an isolated nucleic acid molecule having a nucleic acid sequence comprising or consisting of at least

forty-five, preferably at least 50, more preferably at least 60 and most preferably 75 or more nucleotide residues of the *K.lactis OCH1* gene (SEQ ID NO: 41), and homologs, variants and derivatives thereof. The invention also provides nucleic acid molecules that hybridize under stringent conditions to the above-described nucleic acid molecules. Similarly, isolated polypeptides (including muteins, allelic variants, fragments, derivatives, and analogs) encoded by the nucleic acid molecules of the invention are provided. Also provided are vectors, including expression vectors, which comprise the above nucleic acid molecules of the invention, as described further herein. Similarly, host cells transformed with the nucleic acid molecules or vectors of the invention are provided.

Host Cells of the Invention

[0134] A preferred host cell of the invention is a lower eukaryotic cell, e.g., yeast, a unicellular and multicellular or filamentous fungus. However, a wide variety of host cells are envisioned as being useful in the methods of the invention. Plant cells or insect cells, for instance, may be engineered to express a human-like glycoprotein according to the invention (**Examples 17 and 18**). Likewise, a variety of non-human, mammalian host cells may be altered to express more human-like or otherwise altered glycoproteins using the methods of the invention. As one of skill in the art will appreciate, any eukaryotic host cell (including a human cell) may be used in conjunction with a library of the invention to express one or more chimeric proteins which is targeted to a subcellular location, e.g., organelle, in the host cell where the activity of the protein is modified, and preferably is enhanced. Such a protein is preferably -- but need not necessarily be -- an enzyme involved in protein glycosylation, as exemplified herein. It is envisioned that any protein coding sequence may be targeted and selected for modified activity in a eukaryotic host cell using the methods described herein.

[0135] Lower eukaryotes that are able to produce glycoproteins having the attached *N*-glycan $Man_5GlcNAc_2$ are particularly useful because (a) lacking a high degree of mannosylation (e.g. greater than 8 mannoses per *N*-glycan, or especially 30-40 mannoses), they show reduced immunogenicity in humans; and (b) the *N*-glycan is a substrate for further glycosylation reactions to form an even more

human-like glycoform, e.g., by the action of GlcNAc transferase I (**Fig. 1B**; β1,2 GnTI) to form $GlcNAcMan_5GlcNAc_2$. A yield is obtained of greater than 30 mole %, more preferably a yield of 50-100 mole %, glycoproteins with *N*-glycans having a $Man_5GlcNAc_2$ structure. In a preferred embodiment, more than 50% of the $Man_5GlcNAc_2$ structure is shown to be a substrate for a GnTI activity and can serve as such a substrate *in vivo*.

[0136] Preferred lower eukaryotes of the invention include but are not limited to: *Pichia pastoris, Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis, Pichia methanolica, Pichia sp., Saccharomyces cerevisiae, Saccharomyces sp., Hansenula polymorpha, Kluyveromyces sp., Kluyveromyces lactis, Candida albicans, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reseei, Chrysosporium lucknowense, Fusarium sp. Fusarium gramineum, Fusarium venenatum and Neurospora crassa.*

[0137] In each above embodiment, the method is directed to making a host cell in which the oligosaccharide precursors are enriched in $Man_5GlcNAc_2$. These structures are desirable because they may then be processed by treatment *in vitro*, for example, using the method of Maras and Contreras, U.S. Patent No. 5,834,251. In a preferred embodiment, however, precursors enriched in $Man_5GlcNAc_2$ are processed by at least one further glycosylation reaction *in vivo* -- with glycosidases (e.g., α-mannosidases) and glycosyltransferases (e.g., GnTI) -- to produce human-like N-glycans. Oligosaccharide precursors enriched in $Man_5GlcNAc_2$, for example, are preferably processed to those having $GlcNAcMan_XGlcNAc_2$ core structures, wherein X is 3, 4 or 5, and is preferably 3. N-glycans having a $GlcNAcMan_XGlcNAc_2$ core structure where X is greater than 3 may be converted to $GlcNAcMan_3GlcNAc_2$, e.g., by treatment with an α-1,3 and/or α-1,6 mannosidase activity, where applicable. Additional processing of $GlcNAcMan_3GlcNAc_2$ by treatment with glycosyltransferases (e.g., GnTII) produces $GlcNAc_2Man_3GlcNAc_2$ core structures which may then be modified, as desired, e.g., by *ex vivo* treatment or by heterologous expression in the host cell of

additional glycosylation enzymes, including glycosyltransferases, sugar transporters and mannosidases (see below), to become human-like N-glycans.

[0138] Preferred human-like glycoproteins which may be produced according to the invention include those which comprise *N*-glycans having seven or fewer, or three or fewer, mannose residues; and which comprise one or more sugars selected from the group consisting of galactose, GlcNAc, sialic acid, and fucose.

Formation of complex N-glycans

[0139] Formation of complex N-glycan synthesis is a sequential process by which specific sugar residues are removed and attached to the core oligosaccharide structure. In higher eukaryotes, this is achieved by having the substrate sequentially exposed to various processing enzymes. These enzymes carry out specific reactions depending on their particular location within the entire processing cascade. This "assembly line" consists of ER, early, medial and late Golgi, and the trans Golgi network all with their specific processing environment. To re-create the processing of human glycoproteins in the Golgi and ER of lower eukaryotes, numerous enzymes (e.g. glycosyltransferases, glycosidases, phosphatases and transporters) have to be expressed and specifically targeted to these organelles, and preferably, in a location so that they function most efficiently in relation to their environment as well as to other enzymes in the pathway.

[0140] Because one goal of the methods described herein is to achieve a robust protein production strain that is able to perform well in an industrial fermentation process, the integration of multiple genes into the host cell chromosome involves careful planning. As described above, one or more genes which encode enzymes known to be characteristic of non-human glycosylation reactions are preferably deleted. The engineered cell strain is transformed with a range of different genes encoding desired activities, and these genes are transformed in a stable fashion, thereby ensuring that the desired activity is maintained throughout the fermentation process.

[0141] Any combination of the following enzyme activities may be engineered singly or multiply into the host using methods of the invention: sialyltransferases, mannosidases, fucosyltransferases, galactosyltransferases, GlcNAc transferases,

ER and Golgi specific transporters (e.g. syn- and antiport transporters for UDP-galactose and other precursors), other enzymes involved in the processing of oligosaccharides, and enzymes involved in the synthesis of activated oligosaccharide precursors such as UDP-galactose and CMP-N-acetylneuraminic acid. Preferably, enzyme activities are introduced on one or more nucleic acid molecules (see also below). Nucleic acid molecules may be introduced singly or multiply, e.g., in the context of a nucleic acid library such as a combinatorial library of the invention. It is to be understood, however, that single or multiple enzymatic activities may be introduced into a host cell in any fashion, including but not limited to protein delivery methods and/or by use of one or more nucleic acid molecules without necessarily using a nucleic acid library or combinatorial library of the invention.

Expression Of Glycosyltransferases To Produce Complex N-glycans:

[0142] With DNA sequence information, the skilled artisan can clone DNA molecules encoding GnT activities (e.g., **Examples 3 and 4**). Using standard techniques well-known to those of skill in the art, nucleic acid molecules encoding GnTI, II, III, IV or V (or encoding catalytically active fragments thereof) may be inserted into appropriate expression vectors under the transcriptional control of promoters and other expression control sequences capable of driving transcription in a selected host cell of the invention, e.g., a fungal host such as *Pichia sp.*, *Kluyveromyces sp.* and *Aspergillus sp.*, as described herein, such that one or more of these mammalian GnT enzymes may be actively expressed in a host cell of choice for production of a human-like complex glycoprotein (e.g., **Examples 15, 17** and **18**).

[0143] Several individual glycosyltransferases have been cloned and expressed in *S.cerevisiae* (GalT, GnTI), *Aspergillus nidulans* (GnTI) and other fungi, without however demonstrating the desired outcome of "humanization" on the glycosylation pattern of the organisms (Yoshida, 1995; Schwientek, 1995; Kalsner, 1995). It was speculated that the carbohydrate structure required to accept sugars by the action of such glycosyltransferases was not present in sufficient amounts, which most likely contributed to the lack of complex N-glycan formation.

[0144] A preferred method of the invention provides the functional expression of a GnT, such as GnTI, in the early or medial Golgi apparatus as well as ensuring a sufficient supply of UDP-GlcNAc (e.g., by expression of a UDP-GlcNAc transporter; see below).

Methods for Providing Sugar Nucleotide Precursors to the Golgi Apparatus:

[0145] For a glycosyltransferase to function satisfactorily in the Golgi, the enzyme requires a sufficient concentration of an appropriate nucleotide sugar, which is the high-energy donor of the sugar moiety added to a nascent glycoprotein. In humans, the full range of nucleotide sugar precursors (e.g. UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, CMP-N-acetylneuraminic acid, UDP-galactose, etc.) are generally synthesized in the cytosol and transported into the Golgi, where they are attached to the core oligosaccharide by glycosyltransferases.

[0146] To replicate this process in non-human host cells such as lower eukaryotes, sugar nucleoside specific transporters have to be expressed in the Golgi to ensure adequate levels of nucleoside sugar precursors (Sommers, 1981; Sommers, 1982; Perez, 1987). Nucleotide sugars may be provided to the appropriate compartments, e.g., by expressing in the host microorganism an exogenous gene encoding a sugar nucleotide transporter. The choice of transporter enzyme is influenced by the nature of the exogenous glycosyltransferase being used. For example, a GlcNAc transferase may require a UDP-GlcNAc transporter, a fucosyltransferase may require a GDP-fucose transporter, a galactosyltransferase may require a UDP-galactose transporter, and a sialyltransferase may require a CMP-sialic acid transporter.

[0147] The added transporter protein conveys a nucleotide sugar from the cytosol into the Golgi apparatus, where the nucleotide sugar may be reacted by the glycosyltransferase, e.g. to elongate an *N*-glycan. The reaction liberates a nucleoside diphosphate or monophosphate, e.g. UDP, GDP, or CMP. Nucleoside monophosphates can be directly exported from the Golgi in exchange for nucleoside triphosphate sugars by an antiport mechanism. Accumulation of a nucleoside diphosphate, however, inhibits the further activity of a

glycosyltransferase. As this reaction appears to be important for efficient glycosylation, it is frequently desirable to provide an expressed copy of a gene encoding a nucleotide diphosphatase. The diphosphatase (specific for UDP or GDP as appropriate) hydrolyzes the diphosphonucleoside to yield a nucleoside monosphosphate and inorganic phosphate.

[0148] Suitable transporter enzymes, which are typically of mammalian origin, are described below. Such enzymes may be engineered into a selected host cell using the methods of the invention (see also **Examples 7-10**).

[0149] In another example, α2,3- or α2,6-sialyltransferase caps galactose residues with sialic acid in the trans-Golgi and TGN of humans leading to a mature form of the glycoprotein (**Fig. 1B**). To reengineer this processing step into a metabolically engineered yeast or fungus will require (1) α2,3- or α2,6-sialyltransferase activity and (2) a sufficient supply of CMP-N-acetyl neuraminic acid, in the late Golgi of yeast (**Example 6**). To obtain sufficient α2,3-sialyltransferase activity in the late Golgi, for example, the catalytic domain of a known sialyltransferase (e.g. from humans) has to be directed to the late Golgi in fungi (see above). Likewise, transporters have to be engineered to allow the transport of CMP-N-acetyl neuraminic acid into the late Golgi. There is currently no indication that fungi synthesize or can even transport sufficient amounts of CMP-N-acetyl neuraminic acid into the Golgi. Consequently, to ensure the adequate supply of substrate for the corresponding glycosyltransferases, one has to metabolically engineer the production of CMP-sialic acid into the fungus.

UDP-N-acetylglucosamine

[0150] The cDNA of human UDP-N-acetylglucosamine transporter, which was recognized through a homology search in the expressed sequence tags database (dbEST), has been cloned (Ishida, 1999 *J. Biochem.*126(1): 68-77). The mammalian Golgi membrane transporter for UDP-N-acetylglucosamine was cloned by phenotypic correction with cDNA from canine kidney cells (MDCK) of a recently characterized *Kluyveromyces lactis* mutant deficient in Golgi transport of the above nucleotide sugar (Guillen, 1998). Results demonstrate that the mammalian Golgi UDP-GlcNAc transporter gene has all of the necessary

information for the protein to be expressed and targeted functionally to the Golgi apparatus of yeast and that two proteins with very different amino acid sequences may transport the same solute within the same Golgi membrane (Guillen, 1998).

[0151] Accordingly, one may incorporate the expression of a UDP-GlcNAc transporter in a host cell by means of a nucleic acid construct which may contain, for example: (1) a region by which the transformed construct is maintained in the cell (e.g. origin of replication or a region that mediates chromosomal integration), (2) a marker gene that allows for the selection of cells that have been transformed, including counterselectable and recyclable markers such as *ura3* or *T-urf13* (Soderholm, 2001) or other well characterized selection-markers (e.g., *his4, bla, Sh ble* etc.), (3) a gene or fragment thereof encoding a functional UDP-GlcNAc transporter (e.g. from *K.lactis*, (Abeijon, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93:5963-5968), or from *H.sapiens* (Ishida, 1996), and (4) a promoter activating the expression of the above mentioned localization/catalytic domain fusion construct library.

GDP-Fucose

[0152] The rat liver Golgi membrane GDP-fucose transporter has been identified and purified by Puglielli, L. and C. B. Hirschberg (Puglielli, 1999 *J. Biol. Chem.* 274(50):35596-35600). The corresponding gene has not been identified, however, N-terminal sequencing can be used for the design of oligonucleotide probes specific for the corresponding gene. These oligonucleotides can be used as probes to clone the gene encoding for GDP-fucose transporter.

UDP-Galactose

[0153] Two heterologous genes, *gmal2*(+) encoding alpha 1,2-galactosyltransferase (alpha 1,2 GalT) from *Schizosaccharomyces pombe* and (*hUGT2*) encoding human UDP-galactose (UDP-Gal) transporter, have been functionally expressed in *S.cerevisiae* to examine the intracellular conditions required for galactosylation. Correlation between protein galactosylation and UDP-galactose transport activity indicated that an exogenous supply of UDP-Gal transporter, rather than alpha 1,2 GalT played a key role for efficient

galactosylation in *S.cerevisiae* (Kainuma, 1999 *Glycobiology* 9(2): 133-141). Likewise, an UDP-galactose transporter from *S. pombe* was cloned (Aoki, 1999 *J.Biochem.* 126(5): 940-950; Segawa, 1999 *Febs Letters* 451(3): 295-298).

CMP-N-acetylneuraminic acid (CMP-Sialic acid).

[0154] Human CMP-sialic acid transporter (hCST) has been cloned and expressed in Lec 8 CHO cells (Aoki, 1999; Eckhardt, 1997). The functional expression of the murine CMP-sialic acid transporter was achieved in *Saccharomyces cerevisiae* (Berninsone, 1997). Sialic acid has been found in some fungi, however it is not clear whether the chosen host system will be able to supply sufficient levels of CMP-Sialic acid. Sialic acid can be either supplied in the medium or alternatively fungal pathways involved in sialic acid synthesis can also be integrated into the host genome.

Expression of Diphosphatases:

[0155] When sugars are transferred onto a glycoprotein, either a nucleoside diphosphate or monophosphate is released from the sugar nucleotide precursors. While monophosphates can be directly exported in exchange for nucleoside triphosphate sugars by an antiport mechanism, diphosphonucleosides (e.g. GDP) have to be cleaved by phosphatases (e.g. GDPase) to yield nucleoside monophosphates and inorganic phosphate prior to being exported. This reaction appears to be important for efficient glycosylation, as GDPase from *S.cerevisiae* has been found to be necessary for mannosylation. However, the enzyme only has 10% of the activity towards UDP (Berninsone, 1994). Lower eukaryotes often do not have UDP-specific diphosphatase activity in the Golgi as they do not utilize UDP-sugar precursors for glycoprotein synthesis in the Golgi. *Schizosaccharomyces pombe*, a yeast which adds galactose residues to cell wall polysaccharides (from UDP-galactose), was found to have specific UDPase activity, further suggesting the requirement for such an enzyme (Berninsone, 1994). UDP is known to be a potent inhibitor of glycosyltransferases and the removal of this glycosylation side product is important to prevent glycosyltransferase inhibition in the lumen of the Golgi (Khatara et al. 1974).

Methods For Altering N-Glycans in a Host By Expressing A Targeted Enzymatic Activity From a Nucleic Acid Molecule

[0156] The present invention further provides a method for producing a human-like glycoprotein in a non-human host cell comprising the step of introducing into the cell one or more nucleic acid molecules which encode an enzyme or enzymes for production of the $Man_5GlcNAc_2$ carbohydrate structure. In one preferred embodiment, a nucleic acid molecule encoding one or more mannosidase activities involved in the production of $Man_5GlcNAc_2$ from $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ is introduced into the host. The invention additionally relates to methods for making altered glycoproteins in a host cell comprising the step of introducing into the host cell a nucleic acid molecule which encodes one or more glycosylation enzymes or activities. Preferred enzyme activities are selected from the group consisting of UDP-GlcNAc transferase, UDP-galactosyltransferase, GDP-fucosyltransferase, CMP-sialyltransferase, UDP-GlcNAc transporter, UDP-galactose transporter, GDP-fucose transporter, CMP-sialic acid transporter, and nucleotide diphosphatases. In a particularly preferred embodiment, the host is selected or engineered to express two or more enzymatic activities in which the product of one activity increases substrate levels of another activity, e.g., a glycosyltransferase and a corresponding sugar transporter, e.g., GnTI and UDP-GlcNAc transporter activities. In another preferred embodiment, the host is selected or engineered to expresses an activity to remove products which may inhibit subsequent glycosylation reactions, e.g. a UDP- or GDP-specific diphosphatase activity.

[0157] Preferred methods of the invention involve expressing one or more enzymatic activities from a nucleic acid molecule in a host cell and comprise the step of targeting at least one enzymatic activity to a desired subcellular location (e.g., an organelle) by forming a fusion protein comprising a catalytic domain of the enzyme and a cellular targeting signal peptide, e.g., a heterologous signal peptide which is not normally ligated to or associated with the catalytic domain. The fusion protein is encoded by at least one genetic construct ("fusion construct") comprising a nucleic acid fragment encoding a cellular targeting signal peptide

ligated in the same translational reading frame ("in-frame") to a nucleic acid fragment encoding an enzyme (e.g., glycosylation enzyme), or catalytically active fragment thereof.

[0158] The targeting signal peptide component of the fusion construct or protein is preferably derived from a member of the group consisting of: membrane-bound proteins of the ER or Golgi, retrieval signals, Type II membrane proteins, Type I membrane proteins, membrane spanning nucleotide sugar transporters, mannosidases, sialyltransferases, glucosidases, mannosyltransferases and phosphomannosyltransferases.

[0159] The catalytic domain component of the fusion construct or protein is preferably derived from a glycosidase, mannosidase or a glycosyltransferase activity derived from a member of the group consisting of GnTI, GnTII, GnTIII, GnTIV, GnTV, GnTVI, GalT, Fucosyltransferase and Sialyltransferase. The catalytic domain preferably has a pH optimum within 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the enzyme is localized, or has optimal activity at a pH between 5.1 and 8.0. In a preferred embodiment, the catalytic domain encodes a mannosidase selected from the group consisting of *C. elegans* mannosidase IA, *C. elegans* mannosidase IB, *D. melanogaster* mannosidase IA, *H. sapiens* mannosidase IB, *P. citrinum* mannosidase I, mouse mannosidase IA, mouse mannosidase IB, *A. nidulans* mannosidase IA, *A. nidulans* mannosidase IB, *A. nidulans* mannosidase IC, mouse mannosidase II, *C. elegans* mannosidase II, *H. sapiens* mannosidase II, and mannosidase III.

Selecting a Glycosylation Enzyme: pH Optima and Subcellular Localization

[0160] In one embodiment of the invention, a human-like glycoprotein is made efficiently in a non-human eukaryotic host cell by introducing into a subcellular compartment of the cell a glycosylation enzyme selected to have a pH optimum similar to the pH optima of other enzymes in the targeted subcellular compartment. For example, most enzymes that are active in the ER and Golgi apparatus of *S.cerevisiae* have pH optima that are between about 6.5 and 7.5 (see **Table 3**). Because the glycosylation of proteins is a highly evolved and efficient process, the

internal pH of the ER and the Golgi is likely also in the range of about 6-8. All previous approaches to reduce mannosylation by the action of recombinant mannosidases in fungal hosts, however, have introduced enzymes that have a pH optimum of around pH 5.0 (Martinet et al., 1998, and Chiba et al., 1998). At pH 7.0, the *in vitro* determined activity of those mannosidases is reduced to less than 10%, which is likely insufficient activity at their point of use, namely, the ER and early Golgi, for the efficient *in vivo* production of $Man_5GlcNAc_2$ on N-glycans.

[0161] Accordingly, a preferred embodiment of this invention targets a selected glycosylation enzyme (or catalytic domain thereof), e.g., an α-mannosidase, to a subcellular location in the host cell (e.g., an organelle) where the pH optimum of the enzyme or domain is within 1.4 pH units of the average pH optimum of other representative marker enzymes localized in the same organelle(s). The pH optimum of the enzyme to be targeted to a specific organelle should be matched with the pH optimum of other enzymes found in the same organelle to maximize the activity per unit enzyme obtained. **Table 3** summarizes the activity of mannosidases from various sources and their respective pH optima. **Table 4** summarizes their typical subcellular locations.

Table 3. Mannosidases and their pH optimum.

Source	Enzyme	pH optimum	Reference
Aspergillus saitoi	α-1,2-mannosidase	5.0	Ichishima et al., 1999 Biochem. J. 339(Pt 3):589-597
Trichoderma reesei	α-1,2-mannosidase	5.0	Maras et al., 2000 J. Biotechnol. 77(2-3):255-263
Penicillium citrinum	α-D-1,2-mannosidase	5.0	Yoshida et al., 1993 Biochem. J. 290(Pt 2):349-354
C.elegans	α-1,2-mannosidase	5.5	see **Fig. 11**
Aspergillus nidulans	α-1,2-mannosidase	6.0	Eades and Hintz, 2000
Homo sapiens IA(Golgi)	α-1,2-mannosidase	6.0	
Homo sapiens IB (Golgi)	α-1,2-mannosidase	6.0	
Lepidopteran insect cells	Type I α-1,2-Man_6-mannosidase	6.0	Ren et al., 1995 Biochem. 34(8):2489-2495
Homo sapiens	α-D-mannosidase	6.0	Chandrasekaran et al., 1984 Cancer Res. 44(9):4059-68
Xanthomonas manihotis	α-1,2,3-mannosidase	6.0	U.S. Pat. No. 6,300,113
Mouse IB (Golgi)	α-1,2-mannosidase	6.5	Schneikert and Herscovics, 1994 Glycobiology. 4(4):445-50
Bacillus sp. (secreted)	α-D-1,2-mannosidase	7.0	Maruyama et al., 1994 Carbohydrate Res. 251:89-98

[0162] In a preferred embodiment, a particular enzyme or catalytic domain is targeted to a subcellular location in the host cell by means of a chimeric fusion construct encoding a protein comprising a cellular targeting signal peptide not normally associated with the enzymatic domain. Preferably, an enzyme or domain is targeted to the ER, the early, medial or late Golgi of the trans Golgi apparatus of the host cell.

[0163] In a more preferred embodiment, the targeted glycosylation enzyme is a mannosidase, glycosyltransferase or a glycosidase. In an especially preferred embodiment, mannosidase activity is targeted to the ER or cis Golgi, where the

early reactions of glycosylation occur. While this method is useful for producing a human-like glycoprotein in a non-human host cell, it will be appreciated that the method is also useful more generally for modifying carbohydrate profiles of a glycoprotein in any eukaryotic host cell, including human host cells.

[0164] Targeting sequences which mediate retention of proteins in certain organelles of the host cell secretory pathway are well-known and described in the scientific literature and public databases, as discussed in more detail below with respect to libraries for selection of targeting sequences and targeted enzymes. Such subcellular targeting sequences may be used alone or in combination to target a selected glycosylation enzyme (or catalytic domain thereof) to a particular subcellular location in a host cell, i.e., especially to one where the enzyme will have enhanced or optimal activity based on pH optima or the presence of other stimulatory factors.

[0165] When one attempts to trim high mannose structures to yield $Man_5GlcNAc_2$ in the ER or the Golgi apparatus of a host cell such as *S.cerevisiae,* for example, one may choose any enzyme or combination of enzymes that (1) has a sufficiently close pH optimum (i.e. between pH 5.2 and pH 7.8), and (2) is known to generate, alone or in concert, the specific isomeric $Man_5GlcNAc_2$ structure required to accept subsequent addition of GlcNAc by GnTI. Any enzyme or combination of enzymes that is shown to generate a structure that can be converted to $GlcNAcMan_5GlcNAc_2$ by GnTI *in vitro* would constitute an appropriate choice. This knowledge may be obtained from the scientific literature or experimentally.

[0166] For example, one may determine whether a potential mannosidase can convert $Man_8GlcNAc_2$-2AB (2-aminobenzamide) to $Man_5GlcNAc_2$-AB and then verify that the obtained $Man_5GlcNAc_2$-2AB structure can serve a substrate for GnTI and UDP-GlcNAc to give $GlcNAcMan_5GlcNAc_2$ *in vitro*. Mannosidase IA from a human or murine source, for example, would be an appropriate choice (see, e.g., **Example 11**). Examples described herein utilize 2-aminobenzamide labeled N-linked oligomannose followed by HPLC analysis to make this determination.

Table 4. Cellular location and pH optima of various glycosylation-related enzymes of *S.cerevisiae.*

Gene	Activity	Location	pH optimum	Reference(s)
KTR1	α- 1,2 mannosyltransferase	Golgi	7.0	Romero et al., 1997 Biochem. J. 321(Pt 2):289-295
MNS1	α- 1,2- mannosidase	ER	6.5	
CWH41	glucosidase I	ER	6.8	
--	mannosyltransferase	Golgi	7-8	Lehele and Tanner, 1974 Biochim. Biophys. Acta 350(1):225-235
KRE2	α- 1,2 mannosyltransferase	Golgi	6.5-9.0	Romero et al., 1997

[0167] Accordingly, a glycosylation enzyme such as an α-1,2-mannosidase enzyme used according to the invention has an optimal activity at a pH of between 5.1 and 8.0. In a preferred embodiment, the enzyme has an optimal activity at a pH of between 5.5 and 7.5. The *C. elegans* mannosidase enzyme, for example, works well in the methods of the invention and has an apparent pH optimum of about 5.5). Preferred mannosidases include those listed in **Table 3** having appropriate pH optima, e.g. *Aspergillus nidulans*, *Homo sapiens* IA (Golgi), *Homo sapiens* IB (Golgi), *Lepidopteran* insect cells (IPLB-SF21AE), *Homo sapiens*, mouse IB (Golgi), *Xanthomonas manihotis*, *Drosophila melanogaster* and *C. elegans*.

[0168] The experiment which illustrates the pH optimum for an α-1,2-mannosidase enzyme is described in **Example 14**. A chimeric fusion protein BB27-2 (*Saccharomyces MNN10* (s)/*C. elegans* mannosidase IB Δ31), which leaks into the medium was subjected to various pH ranges to determine the optimal activity of the enzyme. The results of the experiment show that the α-1,2-mannosidase has an optimal pH of about 5.5 for its function (**Fig. 11**).

[0169] In a preferred embodiment, a single cloned mannosidase gene is expressed in the host organism. However, in some cases it may be desirable to express several different mannosidase genes, or several copies of one particular gene, in order to achieve adequate production of $Man_5GlcNAc_2$. In cases where multiple genes are used, the encoded mannosidases preferably all have pH optima

within the preferred range of about 5.1 to about 8.0, or especially between about 5.5 and about 7.5. Preferred mannosidase activities include α-1,2-mannosidases derived from mouse, human, *Lepidoptera*, *Aspergillus nidulans*, or *Bacillus* sp., *C.elegans*, *D.melanogaster*, *P.citrinum*, *X.laevis* or *A.nidulans*.

5

In Vivo Alteration of Host Cell Glycosylation Using a Combinatorial DNA Library

[0170] Certain methods of the invention are preferably (but need not necessarily be) carried out using one or more nucleic acid libraries. An exemplary feature of a combinatorial nucleic acid library of the invention is that it comprises sequences encoding cellular targeting signal peptides and sequences encoding proteins to be targeted (e.g., enzymes or catalytic domains thereof, including but not limited to those which mediate glycosylation).

[0171] In one embodiment, a combinatorial nucleic acid library comprises: (a) at least two nucleic acid sequences encoding different cellular targeting signal peptides; and (b) at least one nucleic acid sequence encoding a polypeptide to be targeted. In another embodiment, a combinatorial nucleic acid library comprises: (a) at least one nucleic acid sequence encoding a cellular targeting signal peptide; and (b) at least two nucleic acid sequences encoding a polypeptide to be targeted into a host cell. As described further below, a nucleic acid sequence derived from (a) and a nucleic acid sequence derived from (b) are ligated to produce one or more fusion constructs encoding a cellular targeting signal peptide functionally linked to a polypeptide domain of interest. One example of a functional linkage is when the cellular targeting signal peptide is ligated to the polypeptide domain of interest in the same translational reading frame ("in-frame").

[0172] In a preferred embodiment, a combinatorial DNA library expresses one or more fusion proteins comprising cellular targeting signal peptides ligated in-frame to catalytic enzyme domains. The encoded fusion protein preferably comprises a catalytic domain of an enzyme involved in mammalian- or human-like modification of *N*-glycans. In a more preferred embodiment, the catalytic domain is derived from an enzyme selected from the group consisting of mannosidases, glycosyltransferases and other glycosidases which is ligated in-frame to one or

more targeting signal peptides. The enzyme domain may be exogenous and/or endogenous to the host cell. A particularly preferred signal peptide is one normally associated with a protein that undergoes ER to Golgi transport.

[0173] The combinatorial DNA library of the present invention may be used for producing and localizing *in vivo* enzymes involved in mammalian- or human-like *N*-glycan modification. The fusion constructs of the combinatorial DNA library are engineered so that the encoded enzymes are localized in the ER, Golgi or the trans-Golgi network of the host cell where they are involved in producing particular *N*-glycans on a glycoprotein of interest. Localization of *N*-glycan modifying enzymes of the present invention is achieved through an anchoring mechanism or through protein-protein interaction where the localization peptide constructed from the combinatorial DNA library localizes to a desired organelle of the secretory pathway such as the ER, Golgi or the trans Golgi network.

[0174] An example of a useful *N*-glycan, which is produced efficiently and in sufficient quantities for further modification by human-like (complex) glycosylation reactions is $Man_5GlcNAc_2$. A sufficient amount of $Man_5GlcNAc_2$ is needed on a glycoprotein of interest for further human-like processing *in vivo* (e.g., more than 30 mole %). The $Man_5GlcNAc_2$ intermediate may be used as a substrate for further *N*-glycan modification to produce $GlcNAcMan_5GlcNAc_2$ (**Fig. 1B**; see above). Accordingly, the combinatorial DNA library of the present invention may be used to produce enzymes which subsequently produce $GlcNAcMan_5GlcNAc_2$, or other desired complex N-glycans, in a useful quantity.

[0175] A further aspect of the fusion constructs produced using the combinatorial DNA library of the present invention is that they enable sufficient and often near complete intracellular *N*-glycan trimming activity in the engineered host cell. Preferred fusion constructs produced by the combinatorial DNA library of the invention encode a glycosylation enzyme, e.g., a mannosidase, which is effectively localized to an intracellular host cell compartment and thereby exhibits very little and preferably no extracellular activity. The preferred fusion constructs of the present invention that encode a mannosidase enzyme are shown to localize where the *N*-glycans are modified, namely, the ER and the Golgi. The fusion enzymes of the present invention are targeted to such particular organelles in the secretory

pathway where they localize and act upon *N*-glycans such as $Man_8GlcNAc_2$ to produce $Man_5GlcNAc_2$ on a glycoprotein of interest.

[0176] Enzymes produced by the combinatorial DNA library of the present invention can modify *N*-glycans on a glycoprotein of interest as shown for K3 or IFN-β proteins expressed in *P.pastoris*, as shown in **Fig. 5** and **Fig. 6**, respectively (see also **Examples 2 and 11**). It is, however, appreciated that other types of glycoproteins, without limitation, including erythropoietin, cytokines such as interferon-α, interferon-β, interferon-γ, interferon-ω, and granulocyte-CSF, coagulation factors such as factor VIII, factor IX, and human protein C, soluble IgE receptor α-chain, IgG, IgG fragments, IgM, interleukins, urokinase, chymase, and urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, osteoprotegerin, α-1 antitrypsin, DNase II, α- feto proteins, AAT, rhTBP-1 (onercept, aka TNF Binding protein 1), TACI-Ig (transmembrane activator and calcium modulator and cyclophilin ligand interactor), FSH (follicle stimulating hormone), GM-CSF, GLP-1 w/ and w/o FC (glucagon like protein 1) IL-1 receptor agonist, sTNFr (enbrel, aka soluble TNF receptor Fc fusion) ATIII, rhThrombin, glucocerebrosidase and CTLA4-Ig (Cytotoxic T Lymphocyte associated Antigen 4 - Ig) may be glycosylated in this way.

Constructing a Combinatorial DNA Library of Fusion Constructs:

[0177] A combinatorial DNA library of fusion constructs features one or more cellular targeting signal peptides ("targeting peptides") generally derived from N-terminal domains of native proteins (e.g., by making C-terminal deletions). Some targeting peptides, however, are derived from the C-terminus of native proteins (e.g. *SEC12*). Membrane-bound proteins of the ER or the Golgi are preferably used as a source for targeting peptide sequences. These proteins have sequences encoding a cytosolic tail (ct), a transmembrane domain (tmd) and a stem region (sr) which are varied in length. These regions are recognizable by protein sequence alignments and comparisons with known homologs and/or other localized proteins (e.g., comparing hydrophobicity plots).

[0178] The targeting peptides are indicated herein as short (s), medium (m) and long (l) relative to the parts of a type II membrane. The targeting peptide sequence indicated as short (s) corresponds to the transmembrane domain (tmd) of the membrane-bound protein. The targeting peptide sequence indicated as long (l) corresponds to the length of the transmembrane domain (tmd) and the stem region (sr). The targeting peptide sequence indicated as medium (m) corresponds to the transmembrane domain (tmd) and approximately half the length of the stem region (sr). The catalytic domain regions are indicated herein by the number of nucleotide deletion with respect to its wild-type glycosylation enzyme.

Sub-libraries

[0179] In some cases a combinatorial nucleic acid library of the invention may be assembled directly from existing or wild-type genes. In a preferred embodiment, the DNA library is assembled from the fusion of two or more sub-libraries. By the in-frame ligation of the sub-libraries, it is possible to create a large number of novel genetic constructs encoding useful targeted protein domains such as those which have glycosylation activities.

Catalytic Domain Sub-Libraries Encoding Glycosylation Activities

[0180] One useful sub-library includes DNA sequences encoding enzymes such as glycosidases (e.g., mannosidases), glycosyltransferases (e.g., fucosyl-transferases, galactosyltransferases, glucosyltransferases), GlcNAc transferases and sialyltransferases. Catalytic domains may be selected from the host to be engineered, as well as from other related or unrelated organisms. Mammalian, plant, insect, reptile, algal or fungal enzymes are all useful and should be chosen to represent a broad spectrum of biochemical properties with respect to temperature and pH optima. In a preferred embodiment, genes are truncated to give fragments some of which encode the catalytic domains of the enzymes. By removing endogenous targeting sequences, the enzymes may then be redirected and expressed in other cellular loci.

[0181] The choice of such catalytic domains may be guided by the knowledge of the particular environment in which the catalytic domain is subsequently to be

active. For example, if a particular glycosylation enzyme is to be active in the late Golgi, and all known enzymes of the host organism in the late Golgi have a certain pH optimum, or the late Golgi is known to have a particular pH, then a catalytic domain is chosen which exhibits adequate, and preferably maximum, activity at that pH, as discussed above.

Targeting Peptide Sequence Sub-Libraries

[0182] Another useful sub-library includes nucleic acid sequences encoding targeting signal peptides that result in localization of a protein to a particular location within the ER, Golgi, or trans Golgi network. These targeting peptides may be selected from the host organism to be engineered as well as from other related or unrelated organisms. Generally such sequences fall into three categories: (1) N-terminal sequences encoding a cytosolic tail (ct), a transmembrane domain (tmd) and part or all of a stem region (sr), which together or individually anchor proteins to the inner (lumenal) membrane of the Golgi; (2) retrieval signals which are generally found at the C-terminus such as the HDEL **(SEQ ID NO: 5)** or KDEL **(SEQ ID NO: 6)** tetrapeptide; and (3) membrane spanning regions from various proteins, e.g., nucleotide sugar transporters, which are known to localize in the Golgi.

[0183] In the first case, where the targeting peptide consists of various elements (ct, tmd and sr), the library is designed such that the ct, the tmd and various parts of the stem region are represented. Accordingly, a preferred embodiment of the sub-library of targeting peptide sequences includes ct, tmd, and/or sr sequences from membrane-bound proteins of the ER or Golgi. In some cases it may be desirable to provide the sub-library with varying lengths of sr sequence. This may be accomplished by PCR using primers that bind to the 5' end of the DNA encoding the cytosolic region and employing a series of opposing primers that bind to various parts of the stem region.

[0184] Still other useful sources of targeting peptide sequences include retrieval signal peptides, e.g. the tetrapeptides HDEL **(SEQ ID NO: 5)** or KDEL **(SEQ ID NO: 6)**, which are typically found at the C-terminus of proteins that are transported retrograde into the ER or Golgi. Still other sources of targeting peptide sequences

include (a) type II membrane proteins, (b) the enzymes listed in **Table 3**, (c) membrane spanning nucleotide sugar transporters that are localized in the Golgi, and (d) sequences referenced in **Table 5**. (The HDEL signal in column 1, cell 8 is shown in **SEQ ID NO: 5**).

Table 5. Sources of useful compartmental targeting sequences

Gene or Sequence	**Organism**	**Function**	**Location of Gene Product**
MNS1	*A.nidulans*	α-1,2-mannosidase	ER
MNS1	*A.niger*	α-1,2-mannosidase	ER
MNS1	*S.cerevisiae*	α-1,2-mannosidase	ER
GLS1	*S.cerevisiae*	glucosidase	ER
GLS1	*A.niger*	glucosidase	ER
GLS1	*A.nidulans*	glucosidase	ER
HDEL at C-terminus	*Universal in fungi*	retrieval signal	ER
SEC12	*S.cerevisiae*	COPII vesicle protein	ER/Golgi
SEC12	*A.niger*	COPII vesicle protein	ER/Golgi
OCH1	*S.cerevisiae*	1,6-mannosyltransferase	Golgi (cis)
OCH1	*P.pastoris*	1,6-mannosyltransferase	Golgi (cis)
MNN9	*S.cerevisiae*	1,6-mannosyltransferase complex	Golgi
MNN9	*A.niger*	undetermined	Golgi
VAN1	*S.cerevisiae*	undetermined	Golgi
VAN1	*A.niger*	undetermined	Golgi
ANP1	*S.cerevisiae*	undetermined	Golgi
HOC1	*S.cerevisiae*	undetermined	Golgi
MNN10	*S.cerevisiae*	undetermined	Golgi
MNN10	*A.niger*	undetermined	Golgi
MNN11	*S.cerevisiae*	undetermined	Golgi (cis)
MNN11	*A.niger*	undetermined	Golgi (cis)
MNT1	*S.cerevisiae*	1,2-mannosyltransferase	Golgi (cis, medial

KTR1	*P.pastoris*	undetermined	Golgi (medial)
KRE2	*P.pastoris*	undetermined	Golgi (medial)
KTR3	*P.pastoris*	undetermined	Golgi (medial)
MNN2	*S.cerevisiae*	1,2-mannosyltransferase	Golgi (medial)
KTR1	*S.cerevisiae*	undetermined	Golgi (medial)
KTR2	*S.cerevisiae*	undetermined	Golgi (medial)
MNN1	*S.cerevisiae*	1,3-mannosyltransferase	Golgi (trans)
MNN6	*S.cerevisiae*	Phosphomannosyltransferase	Golgi (trans)
2,6 ST	*H. sapiens*	2,6-sialyltransferase	trans Golgi network
UDP-Gal T	*S. pombe*	UDP-Gal transporter	Golgi

[0185] In any case, it is highly preferred that targeting peptide sequences are selected which are appropriate for the particular enzymatic activity or activities to function optimally within the sequence of desired glycosylation reactions. For example, in developing a modified microorganism capable of terminal sialylation of nascent *N*-glycans, a process which occurs in the late Golgi in humans, it is desirable to utilize a sub-library of targeting peptide sequences derived from late Golgi proteins. Similarly, the trimming of $Man_8GlcNAc_2$ by an α-1,2-mannosidase to give $Man_5GlcNAc_2$ is an early step in complex *N*-glycan formation in humans (**Fig. 1B**). It is therefore desirable to have this reaction occur in the ER or early Golgi of an engineered host microorganism. A sub-library encoding ER and early Golgi retention signals is used.

[0186] A series of fusion protein constructs (i.e., a combinatorial DNA library) is then constructed by functionally linking one or a series of targeting peptide sequences to one or a series of sequences encoding catalytic domains. In a preferred embodiment, this is accomplished by the in-frame ligation of a sub-library comprising DNA encoding targeting peptide sequences (above) with a sub-library comprising DNA encoding glycosylation enzymes or catalytically active fragments thereof (see below).

[0187] The resulting library comprises synthetic genes encoding targeting peptide sequence-containing fusion proteins. In some cases it is desirable to

provide a targeting peptide sequence at the N-terminus of a fusion protein, or in other cases at the C-terminus. In some cases, targeting peptide sequences may be inserted within the open reading frame of an enzyme, provided the protein structure of individual folded domains is not disrupted. Each type of fusion protein is constructed (in a step-wise directed or semi-random fashion) and optimal constructs may be selected upon transformation of host cells and characterization of glycosylation patterns in transformed cells using methods of the invention.

Generating Additional Sequence Diversity

[0188] The method of this embodiment is most effective when a nucleic acid, e.g., a DNA library transformed into the host contains a large diversity of sequences, thereby increasing the probability that at least one transformant will exhibit the desired phenotype. Single amino acid mutations, for example, may drastically alter the activity of glycoprotein processing enzymes (Romero et al., 2000). Accordingly, prior to transformation, a DNA library or a constituent sub-library may be subjected to one or more techniques to generate additional sequence diversity. For example, one or more rounds of gene shuffling, error prone PCR, *in vitro* mutagenesis or other methods for generating sequence diversity, may be performed to obtain a larger diversity of sequences within the pool of fusion constructs.

Expression Control Sequences

[0189] In addition to the open reading frame sequences described above, it is generally preferable to provide each library construct with expression control sequences, such as promoters, transcription terminators, enhancers, ribosome binding sites, and other functional sequences as may be necessary to ensure effective transcription and translation of the fusion proteins upon transformation of fusion constructs into the host organism.

[0190] Suitable vector components, e.g., selectable markers, expression control sequences (e.g., promoter, enhancers, terminators and the like) and, optionally, sequences required for autonomous replication in a host cell, are selected as a function of which particular host cell is chosen. Selection criteria for suitable

vector components for use in a particular mammalian or a lower eukaryotic host cell are routine. Preferred lower eukaryotic host cells of the invention include *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium lucknowense*, *Fusarium sp. Fusarium gramineum*, *Fusarium venenatum* and *Neurospora crassa*. Where the host is *Pichia pastoris*, suitable promoters include, for example, the *AOX1*, *AOX2*, GAPDH and *P40* promoters.

Selectable Markers

[0191] It is also preferable to provide each construct with at least one selectable marker, such as a gene to impart drug resistance or to complement a host metabolic lesion. The presence of the marker is useful in the subsequent selection of transformants; for example, in yeast the *URA3*, *HIS4*, *SUC2*, *G418*, *BLA*, or *SH BLE* genes may be used. A multitude of selectable markers are known and available for use in yeast, fungi, plant, insect, mammalian and other eukaryotic host cells.

Transformation

[0192] The nucleic acid library is then transformed into the host organism. In yeast, any convenient method of DNA transfer may be used, such as electroporation, the lithium chloride method, or the spheroplast method. In filamentous fungi and plant cells, conventional methods include particle bombardment, electroporation and agrobacterium mediated transformation. To produce a stable strain suitable for high-density culture (e.g., fermentation in yeast), it is desirable to integrate the DNA library constructs into the host chromosome. In a preferred embodiment, integration occurs via homologous recombination, using techniques well-known in the art. For example, DNA library elements are provided with flanking sequences homologous to sequences of the

host organism. In this manner, integration occurs at a defined site in the host genome, without disruption of desirable or essential genes.

[0193] In an especially preferred embodiment, library DNA is integrated into the site of an undesired gene in a host chromosome, effecting the disruption or deletion of the gene. For example, integration into the sites of the *OCH1*, *MNN1*, or *MNN4* genes allows the expression of the desired library DNA while preventing the expression of enzymes involved in yeast hypermannosylation of glycoproteins. In other embodiments, library DNA may be introduced into the host via a nucleic acid molecule, plasmid, vector (e.g., viral or retroviral vector), chromosome, and may be introduced as an autonomous nucleic acid molecule or by homologous or random integration into the host genome. In any case, it is generally desirable to include with each library DNA construct at least one selectable marker gene to allow ready selection of host organisms that have been stably transformed. Recyclable marker genes such as *ura3*, which can be selected for or against, are especially suitable.

Screening and Selection Processes

[0194] After transformation of the host strain with the DNA library, transformants displaying a desired glycosylation phenotype are selected. Selection may be performed in a single step or by a series of phenotypic enrichment and/or depletion steps using any of a variety of assays or detection methods. Phenotypic characterization may be carried out manually or using automated high-throughput screening equipment. Commonly, a host microorganism displays protein *N*-glycans on the cell surface, where various glycoproteins are localized.

[0195] One may screen for those cells that have the highest concentration of terminal GlcNAc on the cell surface, for example, or for those cells which secrete the protein with the highest terminal GlcNAc content. Such a screen may be based on a visual method, like a staining procedure, the ability to bind specific terminal GlcNAc binding antibodies or lectins conjugated to a marker (such lectins are available from E.Y. Laboratories Inc., San Mateo, CA), the reduced ability of specific lectins to bind to terminal mannose residues, the ability to incorporate a radioactively labeled sugar *in vitro*, altered binding to dyes or charged surfaces, or

may be accomplished by using a Fluorescence Assisted Cell Sorting (FACS) device in conjunction with a fluorophore labeled lectin or antibody (Guillen, 1998).

[0196] Accordingly, intact cells may be screened for a desired glycosylation phenotype by exposing the cells to a lectin or antibody that binds specifically to the desired *N*-glycan. A wide variety of oligosaccharide-specific lectins are available commercially (e.g., from EY Laboratories, San Mateo, CA). Alternatively, antibodies to specific human or animal *N*-glycans are available commercially or may be produced using standard techniques. An appropriate lectin or antibody may be conjugated to a reporter molecule, such as a chromophore, fluorophore, radioisotope, or an enzyme having a chromogenic substrate (Guillen et al., 1998. *Proc. Natl. Acad. Sci. USA* 95(14): 7888-7892)).

[0197] Screening may then be performed using analytical methods such as spectrophotometry, fluorimetry, fluorescence activated cell sorting, or scintillation counting. In other cases, it may be necessary to analyze isolated glycoproteins or *N*-glycans from transformed cells. Protein isolation may be carried out by techniques known in the art. In a preferred embodiment, a reporter protein is secreted into the medium and purified by affinity chromatography (e.g. Ni-affinity or glutathione –S-transferase affinity chromatography). In cases where an isolated *N*-glycan is preferred, an enzyme such as endo-β-*N*-acetylglucosaminidase (Genzyme Co., Boston, MA; New England Biolabs, Beverly, MA) may be used to cleave the *N*-glycans from glycoproteins. Isolated proteins or *N*-glycans may then be analyzed by liquid chromatography (e.g. HPLC), mass spectroscopy, or other suitable means. U.S. Patent No. 5,595,900 teaches several methods by which cells with desired extracellular carbohydrate structures may be identified. In a preferred embodiment, MALDI-TOF mass spectrometry is used to analyze the cleaved N-glycans.

[0198] Prior to selection of a desired transformant, it may be desirable to deplete the transformed population of cells having undesired phenotypes. For example, when the method is used to engineer a functional mannosidase activity into cells, the desired transformants will have lower levels of mannose in cellular glycoprotein. Exposing the transformed population to a lethal radioisotope of mannose in the medium depletes the population of transformants having the

undesired phenotype, i.e. high levels of incorporated mannose (Huffaker TC and Robbins PW., *Proc Natl Acad Sci U S A.* 1983 Dec;80(24):7466-70). Alternatively, a cytotoxic lectin or antibody, directed against an undesirable *N*-glycan, may be used to deplete a transformed population of undesired phenotypes (e.g., Stanley P and Siminovitch L. *Somatic Cell Genet* 1977 Jul;3(4):391-405). U.S. Patent No. 5,595,900 teaches several methods by which cells with a desired extracellular carbohydrate structures may be identified. Repeatedly carrying out this strategy allows for the sequential engineering of more and more complex glycans in lower eukaryotes.

[0199] To detect host cells having on their surface a high degree of the human-like N-glycan intermediate $GlcNAcMan_3GlcNAc_2$, for example, one may select for transformants that allow for the most efficient transfer of GlcNAc by GlcNAc Transferase from UDP-GlcNAc in an *in vitro* cell assay. This screen may be carried out by growing cells harboring the transformed library under selective pressure on an agar plate and transferring individual colonies into a 96-well microtiter plate. After growing the cells, the cells are centrifuged, the cells resuspended in buffer, and after addition of UDP-GlcNAc and GnT II, the release of UDP is determined either by HPLC or an enzyme linked assay for UDP. Alternatively, one may use radioactively labeled UDP-GlcNAc and GnT II, wash the cells and then look for the release of radioactive GlcNAc by N-actylglucosaminidase. All this may be carried manually or automated through the use of high throughput screening equipment. Transformants that release more UDP, in the first assay, or more radioactively labeled GlcNAc in the second assay, are expected to have a higher degree of $GlcNAcMan_3GlcNAc_2$ on their surface and thus constitute the desired phenotype. Similar assays may be adapted to look at the N-glycans on secreted proteins as well.

[0200] Alternatively, one may use any other suitable screen such as a lectin binding assay that is able to reveal altered glycosylation patterns on the surface of transformed cells. In this case the reduced binding of lectins specific to terminal mannoses may be a suitable selection tool. *Galantus nivalis* lectin binds specifically to terminal α-1,3 mannose, which is expected to be reduced if sufficient mannosidase II activity is present in the Golgi. One may also enrich for

desired transformants by carrying out a chromatographic separation step that allows for the removal of cells containing a high terminal mannose content. This separation step would be carried out with a lectin column that specifically binds cells with a high terminal mannose content (e.g., *Galantus nivalis* lectin bound to agarose , Sigma, St.Louis, MO) over those that have a low terminal mannose content.

[0201] In addition, one may directly create such fusion protein constructs, as additional information on the localization of active carbohydrate modifying enzymes in different lower eukaryotic hosts becomes available in the scientific literature. For example, it is known that human β1,4-GalTr can be fused to the membrane domain of *MNT*, a mannosyltransferase from *S.cerevisiae*, and localized to the Golgi apparatus while retaining its catalytic activity (Schwientek et al., 1995). If *S.cerevisiae* or a related organism is the host to be engineered one may directly incorporate such findings into the overall strategy to obtain complex N-glycans from such a host. Several such gene fragments in *P.pastoris* have been identified that are related to glycosyltransferases in *S.cerevisiae* and thus could be used for that purpose.

Alteration of Host Cell Glycosylation Using
Fusion Constructs From Combinatorial Libraries

[0202] The construction of a preferred combinatorial DNA library is illustrated schematically in **Fig. 2** and described in **Example 11**. The fusion construct may be operably linked to a multitude of vectors, such as expression vectors well-known in the art. A wide variety of such fusion constructs were assembled using representative activities as shown in **Table 6**. Combinations of targeting peptide/catalytic domains may be assembled for use in targeting mannosidase, glycosyltransferase and glycosidase activities in the ER, Golgi and the trans Golgi network according to the invention. Surprisingly, the same catalytic domain may have no effect to a very profound effect on N-glycosylation patterns, depending on the type of targeting peptide used (see, e.g., **Table 7, Example 11**).

Mannosidase Fusion Constructs

[0203] A representative example of a mannosidase fusion construct derived from a combinatorial DNA library of the invention is **pFB8**, which a truncated *Saccharomyces SEC12*(m) targeting peptide (988-1296 nucleotides of *SEC12* from SwissProt P11655) ligated in-frame to a 187 N-terminal amino acid deletion of a mouse α-mannosidase IA (Genbank AN 6678787). The nomenclature used herein, thus, refers to the targeting peptide/catalytic domain region of a glycosylation enzyme as *Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187. The encoded fusion protein localizes in the ER by means of the *SEC12* targeting peptide sequence while retaining its mannosidase catalytic domain activity and is capable of producing *in vivo* N-glycans having a $Man_5GlcNAc_2$ structure (**Example 11**; **Fig. 6F, Fig. 7B**).

[0204] The fusion construct **pGC5**, *Saccharomyces MNS1*(m)/mouse mannosidase IB Δ99, is another example of a fusion construct having intracellular mannosidase trimming activity (**Example 11**; **Fig. 5D, Fig. 8B**). Fusion construct **pBC18-5** (*Saccharomyces VAN1*(s)/*C. elegans* mannosidase IB Δ80) is yet another example of an efficient fusion construct capable of producing N-glycans having a $Man_5GlcNAc_2$ structure *in vivo*. By creating a combinatorial DNA library of these and other such mannosidase fusion constructs according to the invention, a skilled artisan may distinguish and select those constructs having optimal intracellular trimming activity from those having relatively low or no activity. Methods using combinatorial DNA libraries of the invention are advantageous because only a select few mannosidase fusion constructs may produce a particularly desired *N*-glycan *in vivo*.

[0205] In addition, mannosidase trimming activity may be specific to a particular protein of interest. Thus, it is to be further understood that not all targeting peptide/mannosidase catalytic domain fusion constructs may function equally well to produce the proper glycosylation on a glycoprotein of interest. Accordingly, a protein of interest may be introduced into a host cell transfected with a combinatorial DNA library to identify one or more fusion constructs which express a mannosidase activity optimal for the protein of interest. One skilled in the art will be able to produce and select optimal fusion construct(s) using the combinatorial DNA library approach described herein.

[0206] It is apparent, moreover, that other such fusion constructs exhibiting localized active mannosidase catalytic domains (or more generally, domains of any enzyme) may be made using techniques such as those exemplified in **Example 11** and described herein. It will be a matter of routine experimentation for one skilled in the art to make and use the combinatorial DNA library of the present invention to optimize, for example, $Man_5GlcNAc_2$ production from a library of fusion constructs in a particular expression vector introduced into a particular host cell.

Glycosyltransferase Fusion Constructs

[0207] Similarly, a glycosyltransferase combinatorial DNA library was made using the methods of the invention. A combinatorial DNA library of sequences derived from glycosyltransferase I (GnTI) activities were assembled with targeting peptides and screened for efficient production in a lower eukaryotic host cell of a $GlcNAcMan_5GlcNAc_2$ N-glycan structure on a marker glycoprotein. A fusion construct shown to produce $GlcNAcMan_5GlcNAc_2$ (**pPB104**), *Saccharomyces MNN9*(s)/human GnTI Δ38 was identified (**Example 15**). A wide variety of such GnTI fusion constructs were assembled (**Example 15, Table 10**). Other combinations of targeting peptide/GnTI catalytic domains can readily be assembled by making a combinatorial DNA library. It is also apparent to one skilled in the art that other such fusion constructs exhibiting glycosyltransferase activity may be made as demonstrated in **Example 15**. It will be a matter of routine experimentation for one skilled in the art to use the combinatorial DNA library method described herein to optimize $GlcNAcMan_5GlcNAc_2$ production using a selected fusion construct in a particular expression vector and host cell line.

[0208] As stated above for mannosidase fusion constructs, not all targeting peptide/GnTI catalytic domain fusion constructs will function equally well to produce the proper glycosylation on a glycoprotein of interest as described herein. However, one skilled in the art will be able to produce and select optimal fusion construct(s) using a DNA library approach as described herein. **Example 15** illustrates a preferred embodiment of a combinatorial DNA library comprising targeting peptides and GnTI catalytic domain fusion constructs involved in producing glycoproteins with predominantly $GlcNAcMan_5GlcNAc_2$ structure.

Using Multiple Fusion Constructs to Alter Host Cell Glycosylation

[0209] In another example of using the methods and libraries of the invention to alter host cell glycosylation, a *P.pastoris* strain with an *OCH1* deletion that expresses a reporter protein (K3) was transformed with multiple fusion constructs isolated from combinatorial libraries of the invention to convert high mannose N-glycans to human-like N-glycans (**Example 15**). First, the mannosidase fusion construct **pFB8** (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187) was transformed into a *P.pastoris* strain lacking 1,6 initiating mannosyltransferases activity (i.e. *och1* deletion; **Example 1**). Second, **pPB103** comprising a *K.lactis MNN2-2* gene (Genbank AN AF106080) encoding an UDP-GlcNAc transporter was constructed to increase further production of $GlcNAcMan_5GlcNAc_2$. The addition of the UDP-GlcNAc transporter increased production of $GlcNAcMan_5GlcNAc_2$ significantly in the *P.pastoris* strain as illustrated in **Fig. 10B**. Third, **pPB104** comprising *Saccharomyces MNN9* (s)/human GnTI Δ38 was introduced into the strain. This *P.pastoris* strain is referred to as "**PBP-3**."

[0210] It is understood by one skilled in the art that host cells such as the above-described yeast strains can be sequentially transformed and/or co-transformed with one or more expression vectors. It is also understood that the order of transformation is not particularly relevant in producing the glycoprotein of interest. The skilled artisan recognizes the routine modifications of the procedures disclosed herein may provide improved results in the production of the glycoprotein of interest.

[0211] The importance of using a particular targeting peptide sequence with a particular catalytic domain sequence becomes readily apparent from the experiments described herein. The combinatorial DNA library provides a tool for constructing enzyme fusions that are involved in modifying *N*-glycans on a glycoprotein of interest, which is especially useful in producing human-like glycoproteins. (Any enzyme fusion, however, may be selected using libraries and methods of the invention.) Desired transformants expressing appropriately targeted, active α-1,2-mannosidase produce K3 with *N*-glycans of the structure $Man_5GlcNAc_2$ as shown in **Figs. 5D and 5E**. This confers a reduced molecular

mass to the cleaved glycan compared to the K3 of the parent *OCH1* deletion strain, as was detected by MALDI-TOF mass spectrometry in **Fig. 5C**.

[0212] Similarly, the same approach was used to produce another secreted glycoprotein: IFN-β comprising predominantly $Man_5GlcNAc_2$. The $Man_5GlcNAc_2$ was removed by PNGase digestion (Papac et al. 1998 *Glycobiology* 8, 445-454) and subjected to MALDI-TOF as shown in **Fig. 6A – 6F**. A single prominent peak at 1254 (m/z) confirms Man_5GlcNA_2 production on IFN- β in **Figs. 6E (pGC5)** (*Saccharomyces MNS1*(m)/mouse mannosidase IB Δ99) and **6F (pFB8)** (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187). Furthermore, in the *P.pastoris* strain PBP-3 comprising **pFB8** (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187), **pPB104** (*Saccharomyces MNN9* (s)/human GnTI Δ38) and **pPB103** (*K.lactis MNN2*-2 gene), the hybrid N-glycan $GlcNAcMan_5GlcNAc_2$ [**b**] was detected by MALDI-TOF (**Fig. 10**).

[0213] After identifying transformants with a high degree of mannose trimming, additional experiments were performed to confirm that mannosidase (trimming) activity occurred *in vivo* and was not predominantly the result of extracellular activity in the growth medium (**Example 13; Figs. 7-9**).

Host Cells

[0214] Although the present invention is exemplified using a *P.pastoris* host organism, it is understood by those skilled in the art that other eukaryotic host cells, including other species of yeast and fungal hosts, may be altered as described herein to produce human-like glycoproteins. The techniques described herein for identification and disruption of undesirable host cell glycosylation genes, e.g. *OCH1*, is understood to be applicable for these and/or other homologous or functionally related genes in other eukaryotic host cells such as other yeast and fungal strains. As described in **Example 16**, *och1 mnn1* genes were deleted from *K.lactis* to engineer a host cell leading to N-glycans that are completely converted to $Man_5GlcNAc_2$ by 1,2-mannosidase (**Fig. 12C**).

[0215] The *MNN1* gene was cloned from *K.lactis* as described in **Example 16**. The nucleic acid and deduced amino acid sequences of the *K.lactis MNN1* gene are shown in SEQ ID NOS: 43 and 44, respectively. Using gene-specific primers, a

construct was made to delete the *MNN1* gene from the genome of *K.lactis* (**Example 16**). Host cells depleted in *och1* and *mnn1* activities produce N-glycans having a $Man_9GlcNAc_2$ carbohydrate structure (see, e.g., **Fig. 10**). Such host cells may be engineered further using, e.g., methods and libraries of the invention, to produce mammalian- or human-like glycoproteins.

[0216] Thus, in another embodiment, the invention provides an isolated nucleic acid molecule having a nucleic acid sequence comprising or consisting of at least forty-five, preferably at least 50, more preferably at least 60 and most preferably 75 or more nucleotide residues of the *K.lactis MNN1* gene (SEQ ID NO: 43), and homologs, variants and derivatives thereof. The invention also provides nucleic acid molecules that hybridize under stringent conditions to the above-described nucleic acid molecules. Similarly, isolated polypeptides (including muteins, allelic variants, fragments, derivatives, and analogs) encoded by the nucleic acid molecules of the invention are provided. In addition, also provided are vectors, including expression vectors, which comprise a nucleic acid molecule of the invention, as described further herein. Similarly host cells transformed with the nucleic acid molecules or vectors of the invention are provided.

[0217] Another aspect of the present invention thus relates to a non-human eukaryotic host strain expressing glycoproteins comprising modified *N*-glycans that resemble those made by human-cells. Performing the methods of the invention in species other than yeast and fungal cells is thus contemplated and encompassed by this invention. It is contemplated that a combinatorial nucleic acid library of the present invention may be used to select constructs that modify the glycosylation pathway in any eukaryotic host cell system. For example, the combinatorial libraries of the invention may also be used in plants, algae and insects, and in other eukaryotic host cells, including mammalian and human cells, to localize proteins, including glycosylation enzymes or catalytic domains thereof, in a desired location along a host cell secretory pathway. Preferably, glycosylation enzymes or catalytic domains and the like are targeted to a subcellular location along the host cell secretory pathway where they are capable of functioning, and preferably, where they are designed or selected to function most efficiently.

[0218] As described in **Examples 17 and 18**, plant and insect cells may be engineered to alter the glycosylation of expressed proteins using the combinatorial library and methods of the invention. Furthermore, glycosylation in mammalian cells, including human cells, may also be modified using the combinatorial library and methods of the invention. It may be possible, for example, to optimize a particular enzymatic activity or to otherwise modify the relative proportions of various N-glycans made in a mammalian host cell using the combinatorial library and methods of the invention.

[0219] Examples of modifications to glycosylation which can be affected using a method according to this embodiment of the invention are: (1) engineering a eukaryotic host cell to trim mannose residues from $Man_8GlcNAc_2$ to yield a $Man_5GlcNAc_2$ *N*-glycan; (2) engineering eukaryotic host cell to add an *N*-acetylglucosamine (GlcNAc) residue to $Man_5GlcNAc_2$ by action of GlcNAc transferase I; (3) engineering a eukaryotic host cell to functionally express an enzyme such as an *N*-acetylglucosaminyl Transferase (GnTI, GnTII, GnTIII, GnTIV, GnTV, GnTVI), mannosidase II, fucosyltransferase (FT), galactosyl tranferase (GalT) or a sialyltransferase (ST).

[0220] By repeating the method, increasingly complex glycosylation pathways can be engineered into a target host, such as a lower eukaryotic microorganism. In one preferred embodiment, the host organism is transformed two or more times with DNA libraries including sequences encoding glycosylation activities. Selection of desired phenotypes may be performed after each round of transformation or alternatively after several transformations have occurred. Complex glycosylation pathways can be rapidly engineered in this manner.

Sequential Glycosylation Reactions

In a preferred embodiment, such targeting peptide/catalytic domain libraries are designed to incorporate existing information on the sequential nature of glycosylation reactions in higher eukaryotes. Reactions known to occur early in the course of glycoprotein processing require the targeting of enzymes that catalyze such reactions to an early part of the Golgi or the ER. For example, the trimming of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ by mannosidases is an early step in

complex N-glycan formation. Because protein processing is initiated in the ER and then proceeds through the early, medial and late Golgi, it is desirable to have this reaction occur in the ER or early Golgi. When designing a library for mannosidase I localization, for example, one thus attempts to match ER and early Golgi targeting signals with the catalytic domain of mannosidase I.

Integration Sites

[0221] As one ultimate goal of this genetic engineering effort is a robust protein production strain that is able to perform well in an industrial fermentation process, the integration of multiple genes into the host (e.g., fungal) chromosome preferably involves careful planning. The engineered strain may likely have to be transformed with a range of different genes, and these genes will have to be transformed in a stable fashion to ensure that the desired activity is maintained throughout the fermentation process. As described herein, any combination of various desired enzyme activities may be engineered into the fungal protein expression host, e.g., sialyltransferases, mannosidases, fucosyltransferases, galactosyltransferases, glucosyltransferases, GlcNAc transferases, ER and Golgi specific transporters (e.g. syn and antiport transporters for UDP-galactose and other precursors), other enzymes involved in the processing of oligosaccharides, and enzymes involved in the synthesis of activated oligosaccharide precursors such as UDP-galactose, CMP-N-acetylneuraminic acid. Examples of preferred methods for modifying glycosylation in a lower eukaryotic host cell, such as *Pichia pastoris*, are shown in **Table 6**. (The HDEL and KDEL signal peptides in the second row of the third column are shown in **SEQ ID NOS: 5 and 6**, respectively).

Table 6. Some preferred embodiments for modifying glycosylation in a lower eukaroytic microorganism

Desired Structure	*Suitable Catalytic Activities*	*Suitable Sources of Localization Sequences*	*Suitable Gene Deletions*	*Suitable Transporters and/or Phosphatases*
$Man_5GlcNAc_2$	α-1,2-mannosidase (murine, human, *Bacillus* sp., *A.nidulans*)	Mns1 (N-terminus, *S.cerevisiae*) Och1 (N-terminus, *S.cerevisiae*, *P.pastoris*) Ktr1 Mnn9 Mnt1 (*S.cerevisiae*) KDEL, HDEL (C-terminus)	*OCH1* *MNN4* *MNN6*	none
$GlcNAcMan_5GlcNAc_2$	GlcNAc Transferase I, (human, murine, rat etc.)	Och1 (N-terminus, *S.cerevisiae*, *P.pastoris*) KTR1 (N-terminus) Mnn1 (N-terminus, *S.cerevisiae*) Mnt1 (N-terminus, *S.cerevisiae*) GDPase (N-terminus, *S.cerevisiae*)	*OCH1* *MNN4* *MNN6*	UDP-GlcNAc transporter (human, murine, *K.lactis*) UDPase (human)
$GlcNAcMan_3GlcNAc_2$	mannosidase II	Ktr1 Mnn1 (N-terminus, *S.cerevisiae*) Mnt1(N-terminus, *S.cerevisiae*) Kre2/Mnt1 (*S.cerevisiae*) Kre2 (*P.pastoris*) Ktr1 (*S.cerevisiae*) Ktr1 (*P.pastoris*) Mnn1 (*S.cerevisiae*)	*OCH1* *MNN4* *MNN6*	UDP-GlcNAc transporter (human, murine, *K.lactis*) UDPase (human)
$GlcNAc_{(2-4)}Man_3GlcNAc_2$	GlcNAc Transferase II, III, IV, V (human, murine)	Mnn1 (N-terminus, *S.cerevisiae*) Mnt1 (N-terminus, *S.cerevisiae*) Kre2/Mnt1 (*S.cerevisiae*) Kre2 (*P.pastoris*) Ktr1 (*S.cerevisiae*) Ktr1 (*P.pastoris*) Mnn1 (*S.cerevisiae*)	*OCH1* *MNN4* *MNN6*	UDP-GlcNAc transporter (human, murine, *K.lactis*) UDPase (human)

$Gal_{(1-4)}GlcNAc_{(2-4)}$-$Man_3GlcNAc_2$	β-1,4-Galactosyl transferase (human)	Mnn1 (N-terminus, *S.cerevisiae*) Mnt1(N-terminus, *S.cerevisiae*) Kre2/Mnt1 (*S.cerevisiae*) Kre2 (*P.pastoris*) Ktr1 (*S.cerevisiae*) Ktr1 (*P.pastoris*) Mnn1 (*S.cerevisiae*)	*OCH1* *MNN4* *MNN6*	UDP-Galactose transporter (human, *S.pombe*)
$NANA_{(1-4)}$-$Gal_{(1-4)}GlcNAc_{(2-4)}$-$Man_3GlcNAc_2$	α-2,6-Sialyltransferase (human) α-2,3-Sialyltransferase	KTR1 MNN1 (N-terminus, *S.cerevisiae*) MNT1 (N-terminus, *S.cerevisiae*) Kre2/Mnt1 (*S.cerevisiae*) Kre2 (*P.pastoris*) Ktr1 (*S.cerevisiae*) Ktr1 (*P.pastoris*) MNN1 (*S.cerevisiae*)	*OCH1* *MNN4* *MNN6*	CMP-Sialic acid transporter (human)

[0222] As any strategy to engineer the formation of complex N-glycans into a host cell such as a lower eukaryote involves both the elimination as well as the addition of particular glycosyltransferase activities, a comprehensive scheme will attempt to coordinate both requirements. Genes that encode enzymes that are undesirable serve as potential integration sites for genes that are desirable. For example, 1,6 mannosyltransferase activity is a hallmark of glycosylation in many known lower eukaryotes. The gene encoding alpha-1,6 mannosyltransferase (*OCH1*) has been cloned from *S.cerevisiae* and mutations in the gene give rise to a viable phenotype with reduced mannosylation. The gene locus encoding alpha-1,6 mannosyltransferase activity therefore is a prime target for the integration of genes encoding glycosyltransferase activity. In a similar manner, one can choose a range of other chromosomal integration sites that, based on a gene disruption event in that locus, are expected to: (1) improve the cells ability to glycosylate in a more human-like fashion, (2) improve the cells ability to secrete proteins, (3) reduce proteolysis of foreign proteins and (4) improve other characteristics of the process that facilitate purification or the fermentation process itself.

Target Glycoproteins

[0223] The methods described herein are useful for producing glycoproteins, especially glycoproteins used therapeutically in humans. Glycoproteins having specific glycoforms may be especially useful, for example, in the targeting of therapeutic proteins. For example, mannose-6-phosphate has been shown to direct proteins to the lysosome, which may be essential for the proper function of several enzymes related to lysosomal storage disorders such as Gaucher's, Hunter's, Hurler's, Scheie's, Fabry's and Tay-Sachs disease, to mention just a few. Likewise, the addition of one or more sialic acid residues to a glycan side chain may increase the lifetime of a therapeutic glycoprotein *in vivo* after administration. Accordingly, host cells (e.g., lower eukaryotic or mammalian) may be genetically engineered to increase the extent of terminal sialic acid in glycoproteins expressed in the cells. Alternatively, sialic acid may be conjugated to the protein of interest *in vitro* prior to administration using a sialic acid transferase and an appropriate substrate. Changes in growth medium composition may be employed in addition to the expression of enzyme activities involved in human-like glycosylation to produce glycoproteins more closely resembling human forms (S. Weikert, et al., *Nature Biotechnology,* 1999, 17, 1116-1121; Werner, Noe, et al 1998 *Arzneimittelforschung 48(8):870-880;* Weikert, Papac et al., 1999; Andersen and Goochee 1994 *Cur. Opin. Biotechnol.* 5: 546-549; Yang and Butler 2000 *Biotechnol.Bioengin.* 68(4): 370-380). Specific glycan modifications to monoclonal antibodies (e.g. the addition of a bisecting GlcNAc) have been shown to improve antibody dependent cell cytotoxicity (Umana P., et al. 1999), which may be desirable for the production of antibodies or other therapeutic proteins.

[0224] Therapeutic proteins are typically administered by injection, orally, pulmonary, or other means. Examples of suitable target glycoproteins which may be produced according to the invention include, without limitation: erythropoietin, cytokines such as interferon-α, interferon-β, interferon-γ, interferon-ω, and granulocyte-CSF, coagulation factors such as factor VIII, factor IX, and human protein C, soluble IgE receptor α-chain, IgG, IgG fragments, IgM, interleukins, urokinase, chymase, and urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein,

angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, osteoprotegerin, α-1 antitrypsin, DNase II, α- feto proteins, AAT, rhTBP-1 (onercept, aka TNF Binding protein 1), TACI-Ig (transmembrane activator and calcium modulator and cyclophilin ligand interactor), FSH (follicle stimulating hormone), GM-CSF, GLP-1 w/ and w/o FC (glucagon like protein 1) IL-1 receptor agonist, sTNFr (enbrel, aka soluble TNF receptor Fc fusion) ATIII, rhThrombin, glucocerebrosidase and CTLA4-Ig (Cytotoxic T Lymphocyte associated Antigen 4 - Ig)

[0225] The following are examples which illustrate the compositions and methods of this invention. These examples should not be construed as limiting: the examples are included for the purposes of illustration only.

EXAMPLE 1
Cloning and Disruption of the *OCH1* gene in *P.pastoria*

Generation of an *OCH1* mutant of *P.pastoris:*

A 1215 bp ORF of the *P.pastoris OCH1* gene encoding a putative α-1,6 mannosyltransferase was amplified from *P.pastoris* genomic DNA (strain X-33, Invitrogen, Carlsbad, CA) using the oligonucleotides 5'-ATGGCGAAGGCAGATGGCAGT-3' (SEQ ID NO:7) and 5'-TTAGTCCTTCCAACTTCCTTC-3' (SEQ ID NO:8) which were designed based on the *P.pastoris OCH1* sequence (Japanese Patent Application Publication No. 8-336387). Subsequently, 2685 bp upstream and 1175 bp downstream of the ORF of the *OCH1* gene were amplified from a *P.pastoris* genomic DNA library (Boehm, T. et al. Yeast 1999 May;15(7):563-72) using the internal oligonucleotides 5'-ACTGCCATCTGCCTTCGCCAT-3' (SEQ ID NO:9) in the *OCH1* gene, and 5'-GTAATACGACTCACTATAGGGC-3' T7 (SEQ ID NO:10) and 5'-AATTAACCCTCACTAAAGGG-3' T3 (SEQ ID NO:11) oligonucleotides in the backbone of the library bearing plasmid lambda ZAP II (Stratagene, La Jolla, CA). The resulting 5075 bp fragment was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and designated pBK9.

[0226] After assembling a gene knockout construct that substituted the *OCH1* reading frame with a *HIS4* resistance gene, *P.pastoris* was transformed and

colonies were screened for temperature sensitivity at 37°C. *OCH1* mutants of *S.cerevisiae* are temperature sensitive and are slow growers at elevated temperatures. One can thus identify functional homologs of *OCH1* in *P.pastoris* by complementing an *OCH1* mutant of *S.cerevisiae* with a *P.pastoris* DNA or cDNA library. About 20 temperature sensitive strains were further subjected to a colony PCR screen to identify colonies with a deleted *och1* gene. Several *och1* deletions were obtained.

[0227] The linearized pBK9.1, which has 2.1 kb upstream sequence and 1.5 kb down stream sequence of *OCH1* gene cassette carrying *Pichia HIS4* gene, was transformed into *P.pastoris* BK1 [GS115 (*his4* Invitrogen Corp., San Diego, CA) carrying the human IFN-β gene in the *AOX1* locus] to knock out the wild-type *OCH1* gene. The initial screening of transformants was performed using histidine drop-out medium followed by replica plating to select the temperature sensitive colonies. Twenty out of two hundred histidine-positive colonies showed a temperature sensitive phenotype at 37°C. To exclude random integration of pBK9.1 into the *Pichia* genome, the 20 temperature-sensitive isolates were subjected to colony PCR using primers specific to the upstream sequence of the integration site and to *HIS4* ORF. Two out of twenty colonies were *och1* defective and further analyzed using a Southern blot and a Western blot indicating the functional *och1* disruption by the *och1* knock-out construct. Genomic DNA were digested using two separate restriction enzymes *BglII* and *ClaI* to confirm the *och1* knock-out and to confirm integration at the open reading frame. The Western Blot showed *och1* mutants lacking a discrete band produced in the GS115 wild type at 46.2 kDa.

EXAMPLE 2
Engineering of *P.pastoris* with α-1,2-Mannosidase to Produce $Man_5GlcNAc_2$-Containing IFN-β Precursors

[0228] An α-1,2-mannosidase is required for the trimming of $Man_8GlcNAc_2$ to yield $Man_5GlcNAc_2$, an essential intermediate for complex *N*-glycan formation. While the production of a $Man_5GlcNAc_2$ precursor is essential, it is not necessarily sufficient for the production of hybrid and complex glycans because the specific

isomer of $Man_5GlcNAc_2$ may or may not be a substrate for GnTI. An *och1* mutant of *P.pastoris* is engineered to express secreted human interferon-β under the control of an *aox* promoter. A DNA library is constructed by the in-frame ligation of the catalytic domain of human mannosidase IB (an α-1,2-mannosidase) with a sub-library including sequences encoding early Golgi and ER localization peptides. The DNA library is then transformed into the host organism, resulting in a genetically mixed population wherein individual transformants each express interferon-β as well as a synthetic mannosidase gene from the library. Individual transformant colonies are cultured and the production of interferon is induced by addition of methanol. Under these conditions, over 90% of the secreted protein is glycosylated interferon-β.

[0229] Supernatants are purified to remove salts and low-molecular weight contaminants by C_{18} silica reversed-phase chromatography. Desired transformants expressing appropriately targeted, active α-1,2-mannosidase produce interferon-β including *N*-glycans of the structure $Man_5GlcNAc_2$, which has a reduced molecular mass compared to the interferon-β of the parent strain. The purified interferon-β is analyzed by MALDI-TOF mass spectroscopy and colonies expressing the desired form of interferon-β are identified.

EXAMPLE 3

Generation of an *och1* Mutant Strain Expressing an α-1,2-Mannosidase, GnTI and GnTII for Production of a Human-Like Glycoprotein.

[0230] The 1215 bp open reading frame of the *P.pastoris OCH1* gene as well as 2685 bp upstream and 1175 bp downstream was amplified by PCR (see also WO 02/00879), cloned into the pCR2.1-TOPO vector (Invitrogen) and designated pBK9. To create an *och1* knockout strain containing multiple auxotrophic markers, 100 μg of pJN329, a plasmid containing an *och1::URA3* mutant allele flanked with *Sfi*I restriction sites was digested with *Sfi*I and used to transform *P.pastoris* strain JC308 (Cereghino et al. *Gene* 263 (2001) 159-169) by electroporation. Following incubation on defined medium lacking uracil for 10 days at room temperature, 1000 colonies were picked and re-streaked. URA^+ clones that were unable to grow at 37°C, but grew at room temperature, were

subjected to colony PCR to test for the correct integration of the *och1::URA3* mutant allele. One clone that exhibited the expected PCR pattern was designated YJN153. The Kringle 3 domain of human plasminogen (K3) was used as a model protein. A Neo^R marked plasmid containing the K3 gene was transformed into strain YJN153 and a resulting strain, expressing K3, was named BK64-1.

[0231] Plasmid pPB103, containing the *Kluyveromyces lactis MNN2-2* gene which encodes a Golgi UDP-N-acetylglucosamine transporter was constructed by cloning a blunt *Bgl*II-*Hind*III fragment from vector pDL02 (Abeijon et al. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93:5963-5968) into *Bgl*II and *Bam*HI digested and blunt ended pBLADE-SX containing the *P.pastoris ADE1* gene (Cereghino et al. (2001) *Gene* 263:159-169). This plasmid was linearized with *Eco*NI and transformed into strain BK64-1 by electroporation and one strain confirmed to contain the *MNN2-2* by PCR analysis was named PBP1.

[0232] A library of mannosidase constructs was generated, comprising in-frame fusions of the leader domains of several type I or type II membrane proteins from *S.cerevisiae* and *P.pastoris* fused with the catalytic domains of several α-1,2-mannosidase genes from human, mouse, fly, worm and yeast sources (see, e.g., WO02/00879, incorporated herein by reference). This library was created in a *P.pastoris HIS4* integration vector and screened by linearizing with *Sal*I, transforming by electroporation into strain PBP1, and analyzing the glycans released from the K3 reporter protein. One active construct chosen was a chimera of the 988-1296 nucleotides (C-terminus) of the yeast *SEC12* gene fused with a N-terminal deletion of the mouse α-1,2-mannosidase IA gene (**Fig. 3**), which was missing the 187 nucleotides. A *P.pastoris* strain expressing this construct was named PBP2.

[0233] A library of GnTI constructs was generated, comprising in-frame fusions of the same leader library with the catalytic domains of GnTI genes from human, worm, frog and fly sources (WO 02/00879). This library was created in a *P.pastoris ARG4* integration vector and screened by linearizing with *Aat*II, transforming by electroporation into strain PBP2, and analyzing the glycans released from K3. One active construct chosen was a chimera of the first 120 bp of the *S.cerevisiae MNN9* gene fused to a deletion of the human GnTI gene, which

was missing the first 154 bp. A *P.pastoris* strain expressing this construct was named PBP3.

[0234] A library of GnTII constructs was generated, which comprised in-frame fusions of the leader library with the catalytic domains of GnTII genes from human and rat sources (WO 02/00879). This library was created in a *P.pastoris* integration vector containing the NST^R gene conferring resistance to the drug nourseothricin. The library plasmids were linearized with *Eco*RI, transformed into strain RDP27 by electroporation, and the resulting strains were screened by analysis of the released glycans from purified K3.

Materials for the Following Reactions

[0235] MOPS, sodium cacodylate, manganese chloride, UDP-galactose and CMP-N-acetylneuraminic acid were from Sigma. Trifluoroacetic acid (TFA) was from Sigma/Aldrich, Saint Louis, MO. Recombinant rat α2,6-sialyltransferase from *Spodoptera frugiperda* and β1,4-galactosyltransferase from bovine milk were from Calbiochem (San Diego, CA). Protein N-glycosidase F, mannosidases, and oligosaccharides were from Glyko (San Rafael, CA). DEAE ToyoPearl resin was from TosoHaas. Metal chelating "HisBind" resin was from Novagen (Madison, WI). 96-well lysate-clearing plates were from Promega (Madison, WI). Protein-binding 96-well plates were from Millipore (Bedford, MA). Salts and buffering agents were from Sigma (St. Louis, MO). MALDI matrices were from Aldrich (Milwaukee, WI).

Protein Purification

[0236] Kringle 3 was purified using a 96-well format on a Beckman BioMek 2000 sample-handling robot (Beckman/Coulter Ranch Cucamonga, CA). Kringle 3 was purified from expression media using a C-terminal hexa-histidine tag. The robotic purification is an adaptation of the protocol provided by Novagen for their HisBind resin. Briefly, a 150uL (μL) settled volume of resin is poured into the wells of a 96-well lysate-binding plate, washed with 3 volumes of water and charged with 5 volumes of 50mM NiSO4 and washed with 3 volumes of binding buffer (5mM imidazole, 0.5M NaCl, 20mM Tris-HCL pH7.9). The protein

expression media is diluted 3:2, media/PBS (60mM PO4, 16mM KCl, 822mM NaCl pH7.4) and loaded onto the columns. After draining, the columns are washed with 10 volumes of binding buffer and 6 volumes of wash buffer (30mM imidazole, 0.5M NaCl, 20mM Tris-HCl pH7.9) and the protein is eluted with 6 volumes of elution buffer (1M imidazole, 0.5M NaCl, 20mM Tris-HCl pH7.9). The eluted glycoproteins are evaporated to dryness by lyophilyzation.

Release of N-linked Glycans

[0237] The glycans are released and separated from the glycoproteins by a modification of a previously reported method (Papac, et al. A. J. S. (1998) *Glycobiology* 8, 445-454). The wells of a 96-well MultiScreen IP (Immobilon-P membrane) plate (Millipore) are wetted with 100uL of methanol, washed with 3X150uL of water and 50uL of RCM buffer (8M urea, 360mM Tris, 3.2mM EDTA pH8.6), draining with gentle vacuum after each addition. The dried protein samples are dissolved in 30uL of RCM buffer and transferred to the wells containing 10uL of RCM buffer. The wells are drained and washed twice with RCM buffer. The proteins are reduced by addition of 60uL of 0.1M DTT in RCM buffer for 1hr at 37°C. The wells are washed three times with 300uL of water and carboxymethylated by addition of 60uL of 0.1M iodoacetic acid for 30min in the dark at room temperature. The wells are again washed three times with water and the membranes blocked by the addition of 100uL of 1% PVP 360 in water for 1hr at room temperature. The wells are drained and washed three times with 300uL of water and deglycosylated by the addition of 30uL of 10mM NH_4HCO_3 pH 8.3 containing one milliunit of N-glycanase (Glyko). After 16 hours at 37°C, the solution containing the glycans was removed by centrifugation and evaporated to dryness.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry

[0238] Molecular weights of the glycans were determined using a Voyager DE PRO linear MALDI-TOF (Applied Biosciences) mass spectrometer using delayed extraction. The dried glycans from each well were dissolved in 15uL of water and

0.5uL spotted on stainless steel sample plates and mixed with 0.5uL of S-DHB matrix (9mg/mL of dihydroxybenzoic acid, 1mg/mL of 5-methoxysalicilic acid in 1:1 water/acetonitrile 0.1% TFA) and allowed to dry.

[0239] Ions were generated by irradiation with a pulsed nitrogen laser (337nm) with a 4ns pulse time. The instrument was operated in the delayed extraction mode with a 125ns delay and an accelerating voltage of 20kV. The grid voltage was 93.00%, guide wire voltage was 0.10%, the internal pressure was less than 5 X 10-7 torr, and the low mass gate was 875Da. Spectra were generated from the sum of 100-200 laser pulses and acquired with a 2 GHz digitizer. $Man_5GlcNAc_2$ oligosaccharide was used as an external molecular weight standard. All spectra were generated with the instrument in the positive ion mode. The estimated mass accuracy of the spectra was 0.5%.

EXAMPLE 4
Engineering a Strain to Produce Galactosyltransferase

Galactosyltransferase Reaction

[0240] Approximately 2mg of protein (r-K3:hPg [PBP6-5]) was purified by nickel-affinity chromatography, extensively dialyzed against 0.1% TFA, and lyophilized to dryness. The protein was redissolved in 150μL of 50mM MOPS, 20mM MnCl2, pH7.4. After addition of 32.5μg (533nmol) of UDP-galactose and 4mU of β 1,4-galactosyltransferase, the sample was incubated at 37°C for 18 hours. The samples were then dialyzed against 0.1% TFA for analysis by MALDI-TOF mass spectrometry.

[0241] The spectrum of the protein reacted with galactosyltransferase showed an increase in mass consistent with the addition of two galactose moieties when compared with the spectrum of a similar protein sample incubated without enzyme. Protein samples were next reduced, carboxymethylated and deglycosylated with PNGase F. The recovered N-glycans were analyzed by MALDI-TOF mass spectrometry. The mass of the predominant glycan from the galactosyltransferase reacted protein was greater than that of the control glycan by a mass consistent with the addition of two galactose moieties (325.4 Da).

EXAMPLE 5

Engineering a Strain to Express functional and active Mannosidase II

[0242] To generate a human-like glycoform, a microorganism is engineered to express a mannosidase II enzyme which removes the two remaining terminal mannoses from the structure $GlcNAcMan_5GlcNAc_2$ (see **Fig. 1B**). A DNA library including sequences encoding cis and medial Golgi localization signals is fused in-frame to a library encoding mannosidase II catalytic domains. The host organism is a strain, e.g. a yeast, that is deficient in hypermannosylation (e.g. an *och1* mutant) and provides *N*-glycans having the structure $GlcNAcMan_5GlcNAc_2$ in the Golgi and/or ER. After transformation, organisms having the desired glycosylation phenotype are selected. An *in vitro* assay is used in one method. The desired structure $GlcNAcMan_3GlcNAc_2$ (but not the undesired $GlcNAcMan_5GlcNAc_2$) is a substrate for the enzyme GlcNAc Transferase II (see **Fig. 1B**). Accordingly, single colonies may be assayed using this enzyme *in vitro* in the presence of the substrate, UDP-GlcNAc. The release of UDP is determined either by HPLC or an enzymatic assay for UDP. Alternatively, radioactively labeled UDP-GlcNAc or MALDI-TOF may be used.

[0243] The foregoing *in vitro* assays are conveniently performed on individual colonies using high-throughput screening equipment. Alternatively a lectin binding assay is used. In this case the reduced binding of lectins specific for terminal mannoses allows the selection of transformants having the desired phenotype. For example, *Galantus nivalis* lectin binds specifically to terminal α-1,3-mannose, the concentration of which is reduced in the presence of operatively expressed mannosidase II activity. In one suitable method, *G. nivalis* lectin attached to a solid agarose support (available from Sigma Chemical, St. Louis, MO) is used to deplete the transformed population of cells having high levels of terminal α-1,3-mannose.

EXAMPLE 6

Engineering a Strain to Express Sialyltransferase

[0244] The enzymes α2,3-sialyltransferase and α2,6-sialyltransferase add terminal sialic acid to galactose residues in nascent human *N*-glycans, leading to mature glycoproteins (see "α2,3 ST; α2,6 ST" in **Fig. 1B**). In human cells, the reactions occur in the trans Golgi or TGN. Accordingly, a DNA library is constructed by the in-frame fusion of sequences encoding sialyltransferase catalytic domains with sequences encoding trans Golgi or TGN localization signals (Malissard et al. *Biochem Biophys Res Commun* 2000 Jan 7;267(1):169-73; Borsig et al. *Biochem Biophys Res Commun* 1995 May 5;210(1):14-20). The host organism is a strain, e.g. a yeast, that is deficient in hypermannosylation (e.g., an *och1* mutant), which provides *N*-glycans having terminal galactose residues in the late Golgi or TGN, and provides a sufficient concentration of CMP-sialic acid in the late Golgi or TGN. Following transformation, transformants having the desired phenotype are selected, e.g., using a fluorescent antibody specific for *N*-glycans having a terminal sialic acid. In addition, the strains are engineered to produce the CMP-NANA precursors.

Sialyltransferase Reaction

[0245] After resuspending the (galactosyltransferase reacted) (**Example 4**) proteins in 10μL of 50mM sodium cacodylate buffer pH6.0, 300μg (488nmol) of CMP-N-acetylneuraminic acid (CMP-NANA) dissolved in 15μL of the same buffer, and 5μL (2mU) of recombinant α-2,6 sialyltransferase were added. After incubation at 37^{O}C for 15 hours, an additional 200μg of CMP-NANA and 1mU of sialyltransferase were added. The protein samples were incubated for an additional 8 hours and then dialyzed and analyzed by MALDI-TOF-MS as above. The spectrum of the glycoprotein reacted with sialyltransferase showed an increase in mass when compared with that of the starting material (the protein after galactosyltransferase reaction). The N-glycans were released and analyzed as above. The increase in mass of the two ion-adducts of the predominant glycan was consistent with the addition of two sialic acid residues (580 and 583Da).

EXAMPLE 7
Engineering a strain to Express UDP-GlcNAc Transporter

[0246] The cDNA of human Golgi UDP-GlcNAc transporter has been cloned by Ishida and coworkers. (Ishida, N., et al. 1999 *J. Biochem.*126(1): 68-77). Guillen and coworkers have cloned the canine kidney Golgi UDP-GlcNAc transporter by phenotypic correction of a *Kluyveromyces lactis* mutant deficient in Golgi UDP-GlcNAc transport. (Guillen, E., et al. 1998). Thus a mammalian Golgi UDP-GlcNAc transporter gene has all of the necessary information for the protein to be expressed and targeted functionally to the Golgi apparatus of yeast. These or other cloned transporter genes may be engineered into a host organism to provide UDP-GlcNAc substrates for efficient GnT reactions in the Golgi and/or ER of the host. **Fig. 10B** demonstrates the effect of a strain expressing a *K.lactis* UDP-GlcNAc transporter. In comparison to **Fig. 10A**, which lacks a UDP-GlcNAc transporter, the effect of adding a UDP-GlcNAc transporter shows a dramatic increase in the production of $GlcNAcMan_5GlcNAc_2$.

EXAMPLE 8
Engineering a strain to Express GDP-Fucose Transporter

[0247] The rat liver Golgi membrane GDP-fucose transporter has been identified and purified by Puglielli, L. and C. B. Hirschberg 1999 *J. Biol. Chem.* 274(50):35596-35600. The corresponding gene can be identified using standard techniques, such as *N*-terminal sequencing and Southern blotting using a degenerate DNA probe. The intact gene is then expressed in a host microorganism that also expresses a fucosyltransferase.

EXAMPLE 9
Engineering a strain to Express UDP-Galactose Transporter

[0248] Human UDP-galactose (UDP-Gal) transporter has been cloned and shown to be active in *S.cerevisiae*. (Kainuma, M., et al. 1999 *Glycobiology* 9(2): 133-141). A second human UDP-galactose transporter (hUGT1) has been cloned and functionally expressed in Chinese Hamster Ovary Cells. Aoki, K., et al. 1999 *J.Biochem.* 126(5): 940-950. Likewise, Segawa and coworkers have cloned a UDP-galactose transporter from *Schizosaccharomyces pombe* (Segawa, H., et al. 1999 *Febs Letters* 451(3): 295-298). These or other sequences encoding UDP-galactose transporter activities may be introduced into a host cell directly or may

be used as a component of a sub-library of the invention to engineer a strain having increased UDP-galactose transporter activity.

EXAMPLE 10
Engineering a Strain to Express CMP-Sialic Acid Transporter

[0249] Human CMP-sialic acid transporter (hCST) has been cloned and expressed in Lec 8 CHO cells by Aoki and coworkers (1999). Molecular cloning of the hamster CMP-sialic acid transporter has also been achieved (Eckhardt and Gerardy Schahn 1997 *Eur. J. Biochem.* 248(1): 187-192). The functional expression of the murine CMP-sialic acid transporter was achieved in *Saccharomyces cerevisiae* by Berninsone, P., et al. 1997 *J. Biol.Chem.*272 (19):12616-12619. These or other sequences encoding CMP-sialic acid transporter activities may be introduced into a host cell directly or may be used as a component of a sub-library of the invention to engineer a strain having increased CMP-sialic acid transporter activity.

EXAMPLE 11
Engineering of *P.pastoris* to Produce Man_5GlcNA_2 as the Predominant *N*-Glycan Structure Using a Combinatorial DNA Library.

[0250] An *och1* mutant of *P.pastoris* (see **Examples 1 and 3)** was engineered to express and secrete proteins such as the kringle 3 domain of human plasminogen (K3) under the control of the inducible *AOX1* promoter. The Kringle 3 domain of human plasminogen (K3) was used as a model protein. A DNA fragment encoding the K3 was amplified using Pfu turbo polymerase (Strategene, La Jolla, CA) and cloned into *EcoRI* and *XbaI* sites of pPICZαA (Invitrogen, Carlsbad, CA), resulting in a C-terminal 6- His tag. In order to improve the N-linked glycosylation efficiency of K3 (Hayes et al. 1975 *J. Arch. Biochem. Biophys.* 171, 651-655), Pro_{46} was replaced with Ser_{46} using site-directed mutagenesis. The resulting plasmid was designated pBK64. The correct sequence of the PCR construct was confirmed by DNA sequencing.

[0251] A combinatorial DNA library was constructed by the in-frame ligation of murine α-1,2-mannosidase IB (Genbank AN 6678787) and IA (Genbank AN 6754619) catalytic domains with a sub-library including sequences encoding Cop

II vesicle, ER, and early Golgi localization peptides according to **Table 6**. The combined DNA library was used to generate individual fusion constructs, which were then transformed into the K3 expressing host organism, resulting in a genetically mixed population wherein individual transformants each express K3 as well as a localization signal/mannosidase fusion gene from the library. Individual transformants were cultured and the production of K3 was induced by transfer to a methanol containing medium. Under these conditions, after 24 hours of induction, over 90% of the protein in the medium was K3. The K3 reporter protein was purified from the supernatant to remove salts and low-molecular weight contaminants by Ni-affinity chromatography. Following affinity purification, the protein was desalted by size exclusion chromatography on a Sephadex G10 resin (Sigma, St. Louis, MO) and either directly subjected to MALDI-TOF analysis described below or the *N*-glycans were removed by PNGase digestion as described below (Release of N-glycans) and subjected to MALDI-TOF analysis Miele et al. 1997 *Biotechnol. Appl. Biochem.* 25, 151-157.

[0252] Following this approach, a diverse set of transformants were obtained; some showed no modification of the *N*-glycans compared to the *och1* knockout strain; and others showed a high degree of mannose trimming (**Fig. 5D, 5E**). Desired transformants expressing appropriately targeted, active α-1,2-mannosidase produced K3 with *N*-glycans of the structure $Man_5GlcNAc_2$. This confers a reduced molecular mass to the glycoprotein compared to the K3 of the parent *och1* deletion strain, a difference which was readily detected by MALDI-TOF mass spectrometry (**Fig. 5**). **Table 7** indicates the relative $Man_5GlcNAc_2$ production levels.

Table 7. A representative combinatorial DNA library of localization sequences/catalytic domains exhibiting relative levels of $Man_5GlcNAc_2$ production.

		Targeting peptide sequences				
Catalytic		*MNS1*(s)	*MNS1*(m)	*MNS1*(l)	*SEC12*(s)	*SEC12*(m)
	Mouse mannosidase 1A Δ187	FB4 ++	FB5 +	FB6 -	FB7 ++	FB8 ++++
	Mouse mannosidase 1B Δ58	GB4 ++	GB5 +	GB6 +	GB7 ++	GB8 +

	Mouse mannosidase 1B Δ99	GC4 -	GC5 +++	GC6 +	GC7 +	GC8 +
	Mouse mannosidase 1B Δ170	GD4 -	GD5 -	GD6 -	GD7 +	GD8 +

Table 8. Another combinatorial DNA library of localization sequences/catalytic domains exhibiting relative levels of $Man_5GlcNAc_2$ production.

		Targeting peptide sequences					
Catalytic Domains		***VAN1*(s)**	***VAN1*(m)**	***VAN1*(l)**	***MNN10*(s)**	***MNN10*(m)**	***MNN10*(l)**
	***C. elegans* mannosidase 1B Δ80**	BC18-5 +++++	BC19 ++++	BC20 +++	BC27 +++++	BC28 +++++	BC29 +++
	***C. elegans* mannosidase 1B Δ31**	BB18 +++++	BB19 +++++	BB20 ++++	BB18 +++++	BB19 +++++	BB20 ++++

[0253] Targeting peptides were selected from *MNS* I (SwissProt P32906) in *S.cerevisiae* (long, medium and short) (see supra Nucleic Acid Libraries; Combinatorial DNA Library of Fusion Constructs) and *SEC12* (SwissProt P11655) in *S.cerevisiae* (988-1140 nucleotides: short) and (988-1296: medium). Although majority of the targeting peptide sequences were N-terminal deletions, some targeting peptide sequences, such as *SEC12* were C-terminal deletions. Catalytic domains used in this experiment were selected from mouse mannosidase 1A with a 187 amino acid N-terminal deletion; and mouse mannosidase 1B with a 58, 99 and 170 amino acid deletion. The number of (+)s, as used herein, indicates the relative levels of Man_5GlcNA_2 production. The notation (-) indicates no apparent production of Man_5GlcNA_2. The notation (+) indicates less than 10% production of Man_5GlcNA_2. The notation (++) indicates about 10-20% production of Man_5GlcNA_2. The notation with (+++) indicates about 20-40% production of Man_5GlcNA_2. The notation with (++++) indicates about 50% production of Man_5GlcNA_2. The notation with (+++++) indicates greater than 50% production of Man_5GlcNA_2.

Table 9 shows relative amount of $Man_5GlcNAc_2$ on secreted K3. Six hundred and eight (608) different strains of *P.pastoris*, Δ*och1* were generated by transforming them with a single construct of a combinatorial genetic library that was generated by fusing nineteen (19) α-1,2 mannosidase catalytic domains to thirty-two (32) fungal ER, and cis-Golgi leaders.

Table 9

Amount of $Man_5GlcNAc_2$ on secreted K3 (% of total glycans)	Number of constructs (%)
N.D.*	19 (3.1)
0-10%	341 (56.1)
10-20%	50 (8.2)
20-40&	75 (12.3)
40-60%	72 (11.8)
More than 60%	51 (8.4) †
Total	608 (100)

* Several fusion constructs were not tested because the corresponding plasmids could not be propagated in *E.coli* prior to transformation into *P.pastoris*.

† Clones with the highest degree of $Man_5GlcNAc_2$ trimming (30/51) were further analyzed for mannosidase activity in the supernatant of the medium. The majority (28/30) displayed detectable mannosidase activity in the supernatant (e.g. **Fig. 4B**). Only two constructs displayed high $Man_5GlcNAc_2$ levels, while lacking mannosidase activity in the medium (e.g. **Fig. 4C**).

[0254] **Table 7** shows two constructs **pFB8** and **pGC5**, among others, displaying Man_5GlcNA_2. **Table 8** shows a more preferred construct, **pBC18-5**, a *S.cerevisiae* *VAN1*(s) targeting peptide sequence (from SwissProt 23642) ligated in-frame to a *C. elegans* mannosidase IB (Genbank AN CAA98114) 80 amino acid N-terminal deletion (*Saccharomyces* Van1(s)/ *C.elegans* mannosidase IB Δ80). This fusion construct also produces a predominant Man_5GlcNA_2 structure, as shown in **Fig. 5E**. This construct was shown to produce greater than 50% Man_5GlcNA_2 (+++++).

Generation of a combinatorial localization/mannosidase library:

[0255] Generating a combinatorial DNA library of α-1,2-mannosidase catalytic domains fused to targeting peptides required the amplification of mannosidase domains with varying lengths of *N*-terminal deletions from a number of organisms. To approach this goal, the full length open reading frames (ORFs) of α-1,2-mannosidases were PCR amplified from either cDNA or genomic DNA obtained from the following sources: *Homo sapiens, Mus musculus, Drosophila melanogaster, Caenorhabditis elegans, Aspergillus nidulans* and *Penicillium citrinum*. In each case, DNA was incubated in the presence of oligonucleotide primers specific for the desired mannosidase sequence in addition to reagents required to perform the PCR reaction. For example, to amplify the ORF of the *M. musculus* α-1,2-mannosidase IA, the 5'-primer ATGCCCGTGGGGGGCCTGTTGCCGCTCTTCAGTAGC (SEQ ID NO:12) and the 3'-primer TCATTTCTCTTTGCCATCAATTTCCTTCTTCTGTTCACGG (SEQ ID NO:13) were incubated in the presence of Pfu DNA polymerase (Stratagene, La Jolla, CA) and amplified under the conditions recommended by Stratagene using the cycling parameters: 94°C for 1min (1 cycle); 94°C for 30 sec, 68°C for 30 sec, 72°C for 3min (30 cycles). Following amplification the DNA sequence encoding the ORF was incubated at 72 °C for 5 min with 1U Taq DNA polymerase (Promega, Madison, WI) prior to ligation into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and transformed into TOP10 chemically competent *E. coli*, as recommended by Invitrogen. The cloned PCR product was confirmed by ABI sequencing using primers specific for the mannosidase ORF.

[0256] To generate the desired N-terminal truncations of each mannosidase, the complete ORF of each mannosidase was used as the template in a subsequent round of PCR reactions wherein the annealing position of the 5'-primer was specific to the 5'-terminus of the desired truncation and the 3'-primer remained specific for the original 3'-terminus of the ORF. To facilitate subcloning of the truncated mannosidase fragment into the yeast expression vector, pJN347 (**Fig. 2C**) *AscI* and *PacI* restriction sites were engineered onto each truncation product, at the 5'- and 3'-termini respectively. The number and position of the *N*-terminal truncations generated for each mannosidase ORF depended on the position of the transmembrane (TM) region in relation to the catalytic domain (CD). For instance,

if the stem region located between the TM and CD was less than 150bp, then only one truncation for that protein was generated. If, however, the stem region was longer than 150bp then either one or two more truncations were generated depending on the length of the stem region.

[0257] An example of how truncations for the *M. musculus* mannosidase IA (Genbank AN 6678787) were generated is described herein, with a similar approach being used for the other mannosidases. **Fig. 3** illustrates the ORF of the *M. musculus* α-1,2-mannosidase IA with the predicted transmembrane and catalytic domains being highlighted in bold. Based on this structure, three 5'-primers were designed (annealing positions underlined in **Fig. 3**) to generate the Δ65-, Δ105- and Δ187-N-terminal deletions. Using the Δ65 N-terminal deletion as an example the 5'-primer used was 5'-**GGCGCGCC**GACTCCTCCAAGCTGCTCAGCGGGGTCCTGTTCCAC-3' (SEQ ID NO:14) (with the *AscI* restriction site highlighted in bold) in conjunction with the 3'-primer 5'-CCT**TAATTAA**TCATTTCTCTTTGCCATCAATTTCCTTCTTCTGTTCACGG-3' (SEQ ID NO:15) (with the *PacI* restriction site highlighted in bold). Both of these primers were used to amplify a 1561 bp fragment under the conditions outlined above for amplifying the full length *M. musculus* mannosidase 1A ORF. Furthermore, like the product obtained for the full length ORF, the truncated product was also incubated with Taq DNA polymerase, ligated into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), transformed into TOP10 and ABI sequenced. After having amplified and confirmed the sequence of the truncated mannosidase fragment, the resulting plasmid, pCR2.1-Δ65mMannIA, was digested with *AscI* and *PacI* in New England Biolabs buffer #4 (Beverly, MA) for 16h at 37°C. In parallel, the pJN347 (**Fig. 2C**) was digested with the same enzymes and incubated as described above. Post-digestion, both the pJN347 (**Fig. 2C**) back-bone and the truncated catalytic domain were gel extracted and ligated using the Quick Ligation Kit (New England Biolabs, Beverly, MA), as recommended by the manufacturers, and transformed into chemically competent DH5α cells (Invitrogen, Carlsbad, CA). Colony PCR was used to confirm the generation of the pJN347-mouse Mannosidase IAΔ65 construct.

[0258] Having generated a library of truncated α-1,2-mannosidase catalytic domains in the yeast expression vector pJN347 (**Fig. 2C**) the remaining step in generating the targeting peptide/catalytic domain library was to clone in-frame the targeting peptide sequences (**Fig. 2**). Both the pJN347-mannosidase constructs (**Fig. 2D**) and the pCR2.1TOPO-targeting peptide constructs (**Fig. 2B**) such as were incubated overnight at 37°C in New England Biolabs buffer #4 in the presence of the restriction enzymes *NotI* and *AscI*. Following digestion, both the pJN347-mannosidase back-bone and the targeting peptide regions were gel-extracted and ligated using the Quick Ligation Kit (New England Biolabs, Beverly, MA), as recommended by the manufacturers, and transformed into chemically competent DH5α cells (Invitrogen, Carlsbad, CA). Subsequently, the pJN347-targeting peptide/mannosidase constructs were ABI sequenced to confirm that the generated fusions were in-frame. The estimated size of the final targeting peptide/alpha-1,2-mannosidase library contains over 1300 constructs generated by the approach described above. **Fig. 2** illustrates construction of the combinatorial DNA library.

Engineering a *P.pastoris OCH1* knock-out strain with multiple auxotrophic markers.

[0259] The first step in plasmid construction involved creating a set of universal plasmids containing DNA regions of the *KEX1* gene of *P.pastoris* (Boehm *et al.* Yeast 1999 May;15(7):563-72) as space holders for the 5' and 3' regions of the genes to be knocked out. The plasmids also contained the *S.cerevisiae* Ura-blaster (Alani et al., *Genetics* 116, 541-545. 1987) as a space holder for the auxotrophic markers, and an expression cassette with a multiple cloning site for insertion of a foreign gene. A 0.9-kb fragment of the *P.pastoris* KEX1-5' region was amplified by PCR using primers GGCGAGCTCGGCCTACCCGGCCAAGGCTGAGATCATTTGTCCAGCTTCA GA (SEQ ID NO:16) and GCCCACGTCGACGGATCCGTTTAAACATCGATTGGAGAGGCTGACACC GCTACTA (SEQ ID NO:17) and *P.pastoris* genomic DNA as a template and cloned into the *SacI*, *SalI* sites of pUC19 (New England Biolabs, Beverly, MA).

The resulting plasmid was cut with *BamHI* and *SalI*, and a 0.8-kb fragment of the *KEX1*-3' region that had been amplified using primers CGGGATCCACTAGTATTTAAATCATATGTGCGAGTGTACAACTCTTCCC ACATGG (SEQ ID NO:18) and GGACGCGTCGACGGCCTACCCGGCCGTACGAGGAATTTCTCGG ATGACTCTTTTC (SEQ ID NO:19) was cloned into the open sites creating pJN262. This plasmid was cut with *BamHI* and the 3.8-kb *BamHI, BglII* fragment of pNKY51 (Alani *et al.* 1987) was inserted in both possible orientations resulting in plasmids pJN263 (**Fig. 4A**) and pJN284 (**Fig. 4B**).

[0260] An expression cassette was created with *NotI* and *PacI* as cloning sites. The GAPDH promoter of *P.pastoris* was amplified using primers CGGGATCCCTCGAGAGATCTTTTTTGTAGAAATGTCTTGGTGCCT (SEQ ID NO:20) and GGACATGCATGCACTAGTGCGGCCGCCACGTGATAGTTGTTCA ATTGATTGAAATAGGGACAA (SEQ ID NO:21) and plasmid pGAPZ-A (Invitrogen) as template and cloned into the *BamHI, SphI* sites of pUC19 (New England Biolabs, Beverly, MA) (**Fig. 4B**). The resulting plasmid was cut with *SpeI* and *SphI* and the CYC1 transcriptional terminator region ("TT") that had been amplified using primers CCTTGCTAGCTTAATTAACCGCGGCACGTCCGACGGCGGCCCA CGGGTCCCA (SEQ ID NO:22) and GGACATGCATGCGGATCCCTTAAGAGCCGGCAGCTTGCAAATT AAAGCCTTCGAGCGTCCC (SEQ ID NO:23) and plasmid pPICZ-A (Invitrogen) as a template was cloned into the open sites creating pJN261 (**Fig. 4B**).

[0261] A knockout plasmid for the *P.pastoris OCH1* gene was created by digesting pJN263 with *SalI* and *SpeI* and a 2.9-kb DNA fragment of the *OCH1*-5' region, which had been amplified using the primers GAACCACGTCGACGGCCATTGCGGCCAAAACCTTTTTTCCTATT CAAACACAAGGCATTGC (SEQ ID NO:24) and CTCCAATACTAGTCGAAGATTATCTTCTACGGTGCCTGGACTC (SEQ ID NO:25) and *P.pastoris* genomic DNA as a template, was cloned into the open sites

(**Fig. 4C**). The resulting plasmid was cut with *EcoRI* and *PmeI* and a 1.0-kb DNA fragment of the *OCH1*-3' region that had been generated using the primers TGGAAGGTTTAAACAAAGCTAGAGTAAAATAGATATAGCGAG ATTAGAGAATG (SEQ ID NO:26) and AAGAATTCGGCTGGAAGGCCTTGTACCTTGATGTAGTTCCCGTT TTCATC (SEQ ID NO:27) was inserted to generate pJN298 (**Fig. 4C**). To allow for the possibility to simultaneously use the plasmid to introduce a new gene, the *BamHI* expression cassette of pJN261 (**Fig. 4B**) was cloned into the unique *BamHI* site of pJN298 (**Fig. 4C**) to create pJN299 (**Fig. 4E**).

[0262] The *P.pastoris* Ura3-blaster cassette was constructed using a similar strategy as described in Lu. P., *et al.* 1998 (Cloning and disruption of the β-isopropylmalate dehydrogenase gene (Leu2) of *Pichia stipidis* with *URA3* and recovery of the double auxotroph. Appl. Microbiol. Biotechnol. 49, 141-146.) A 2.0-kb *PstI, SpeI* fragment of *P.pastoris URA3* was inserted into the *PstI, XbaI* sites of pUC19 (New England Biolabs, Beverly, MA) to create pJN306 (**Fig. 4D**). Then a 0.7-kb *SacI, PvuII* DNA fragment of the *lacZ* open reading frame was cloned into the *SacI, SmaI* sites to yield pJN308 (**Fig. 4D**). Following digestion of pJN308 (**Fig. 4D**) with *PstI*, and treatment with T4 DNA polymerase, the *SacI - PvuII* fragment from *lacZ* that had been blunt-ended with T4 DNA polymerase was inserted generating pJN315 (**Fig. 4D**). The *lacZ*/URA3 cassette was released by digestion with *SacI* and *SphI*, blunt ended with T4 DNA polymerase and cloned into the backbone of pJN299 that had been digested with *PmeI* and *AflII* and blunt ended with T4 DNA polymerase. The resulting plasmid was named pJN329 (**Fig. 4E**).

[0263] A *HIS4* marked expression plasmid was created by cutting pJN261 (**Fig. 4F**) with EcoICRI (**Fig. 4F**). A 2.7kb fragment of the *Pichia pastoris HIS4* gene that had been amplified using the primers GCCCAAGCCGGCCTTAAGGGATCTCCTGATGACTGACTCACTGATAATA AAAATACGG (SEQ ID NO:28) and GGGCGCGTATTTAAATACTAGTGGATCTATCGAATCTAAATGTAAGTTA AAATCTCTAA (SEQ ID NO:29) cut with *NgoMIV* and *SwaI* and then blunt-ended using T4 DNA polymerase, was then ligated into the open site. This

plasmid was named pJN337 (**Fig. 4F**). To construct a plasmid with a multiple cloning site suitable for fusion library construction, pJN337 was cut with *NotI* and *PacI* and the two oligonucleotides GGCCGCCTGCAGATTTAAATGAATTCGGCGCGCCTTAAT (SEQ ID NO:30) and TAAGGCGCGCCGAATTCATTTAAATCTGCAGGGC (SEQ ID NO:31), that had been annealed *in vitro* were ligated into the open sites, creating pJN347 (**Fig. 4F**).

[0264] To create an *och1* knockout strain containing multiple auxotrophic markers, 100 µg of pJN329 was digested with *SfiI* and used to transform *P.pastoris* strain JC308 (Cereghino et al. *Gene* 263 (2001) 159-169) by electroporation. Following transformation, the URA dropout plates were incubated at room temperature for 10 days. One thousand (1000) colonies were picked and restreaked. All 1000 clones were then streaked onto 2 sets of URA dropout plates. One set was incubated at room temperature, whereas the second set was incubated at 37°C. The clones that were unable to grow at 37°C, but grew at room temperature, were subjected to colony PCR to test for the correct *OCHI* knockout. One clone that showed the expected PCR signal (about 4.5 kb) was designated YJN153.

Example 12

Characterization of the Combinatorial DNA Library

[0265] Positive transformants screened by colony PCR confirming integration of the mannosidase construct into the *P.pastoris* genome were subsequently grown at room temperature in 50ml BMGY buffered methanol-complex medium consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base, 4 X 10^{-5}% biotin, and 1% glycerol as a growth medium) until OD_{600nm} 2-6 at which point they were washed with 10ml BMMY (buffered methanol-complex medium consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base, 4 X 10^{-5}% biotin, and 1.5% methanol as a growth medium) media prior to induction of the reporter protein for 24 hours at room temperature in 5ml BMMY. Consequently, the reporter protein was isolated and analyzed by mass spectrophotometry and HPLC to characterize its glycan structure. Using the targeting peptides in **Table 6**,

mannosidase catalytic domains localized to either the ER or the Golgi showed significant level of trimming of a glycan predominantly containing $Man_8GlcNAc_2$ to a glycan predominantly containing $Man_5GlcNAc_2$. This is evident when the glycan structure of the reporter glycoprotein is compared between that of *P.pastoris och1* knock-out in **Figs. 5C, 6C** and the same strain transformed with *M. musculus* mannosidase constructs as shown in **Figs. 5D, 5E, 6D – 6F**. **Figs. 5 and 6** show expression of constructs generated from the combinatorial DNA library which show significant mannosidase activity in *P.pastoris*. Expression of pGC5 (*Saccharomyces MNS1*(m)/mouse mannosidase IB Δ99) (**Fig. 5D, 6E**) produced a protein which has approximately 30% of all glycans trimmed to $Man_5GlcNAc_2$, while expression of pFB8 (*Saccharomyces SEC12*(m)/mouse mannosidase IA Δ187) (**Fig. 6F**) produced approximately 50% $Man_5GlcNAc_2$ and expression of pBC18-5 (*Saccharomyces VAN1*(s)/ *C. elegans* mannosidase IB Δ80) (**Fig. 5E**) produced 70% $Man_5GlcNAc_2$.

Release of *N*-glycans

[0266] The glycans were released and separated from the glycoproteins by a modification of a previously reported method (Papac et al. 1998 *Glycobiology* 8, 445-454). After the proteins were reduced and carboxymethylated and the membranes blocked, the wells were washed three time with water. The protein was deglycosylated by the addition of 30 µl of 10 mM NH4HCO3 pH 8.3 containing one milliunit of N-glycanase (Glyko, Novato, CA). After 16 hr at 37°C, the solution containing the glycans was removed by centrifugation and evaporated to dryness.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry

[0267] After the *N*-glycans were released by PNGase digestion, they were analyzed by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry. Molecular weights of the glycans were determined using a Voyager DE PRO linear MALDI-TOF (Applied Biosciences) mass spectrometer using delayed extraction. The dried glycans from each well were dissolved in 15 µl of

water and 0.5 µl was spotted on stainless steel sample plates and mixed with 0.5 µl of S-DHB matrix (9 mg/ml of dihydroxybenzoic acid, 1 mg/ml of 5-methoxysalicilic acid in 1:1 water/acetonitrile 0.1% TFA) and allowed to dry. Ions were generated by irradiation with a pulsed nitrogen laser (337 nm) with a 4 ns pulse time. The instrument was operated in the delayed extraction mode with a 125 ns delay and an accelerating voltage of 20 kV. The grid voltage was 93.00%, guide wire voltage was 0.1%, the internal pressure was less than 5 X 10-7 torr, and the low mass gate was 875 Da. Spectra were generated from the sum of 100-200 laser pulses and acquired with a 500 MHz digitizer. $Man_5GlcNAc_2$ oligosaccharide was used as an external molecular weight standard. All spectra were generated with the instrument in the positive ion mode.

Example 13: Trimming *in vivo* by alpha-1,2-mannosidase

[0268] To ensure that the novel engineered strains of **Example 11** in fact produced the desired $Man_5GlcNAc_2$ structure *in vivo,* cell supernatants were tested for mannosidase activity (see **Figs. 7 – 9**). For each construct/host strain described below, HPLC was performed at 30°C with a 4.0mm x 250 mm column of Altech (Avondale, PA, USA) Econosil-NH_2 resin (5µm) at a flow rate of 1.0 ml/min for 40 min. In **Figs. 7 and 8**, degradation of the standard $Man_9GlcNAc_2$ [b] was shown to occur resulting in a peak which correlates to $Man_8GlcNAc_2$. In **Fig. 7**, the $Man_9GlcNAc_2$ [b] standard eluted at 24.61 min and $Man_5GlcNAc_2$ [a] eluted at 18.59 min. In **Fig. 8**, $Man_9GlcNAc_2$ eluted at 21.37 min and $Man_5GlcNAc_2$ at 15.67 min. In **Fig. 9**, the standard $Man_8GlcNAc_2$ [b] was shown to elute at 20.88 min.

[0269] *P.pastoris* cells comprising plasmid **pFB8** (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187) were grown at 30°C in BMGY to an OD600 of about 10. Cells were harvested by centrifugation and transferred to BMMY to induce the production of K3 (kringle 3 from human plasminogen) under control of an *AOX1* promoter. After 24 hours of induction, cells were removed by centrifugation to yield an essentially clear supernatant. An aliquot of the supernatant was removed for mannosidase assays and the remainder was used for the recovery of secreted soluble K3. A single purification step using CM-

sepharose chromatography and an elution gradient of 25mM NaAc, pH5.0 to 25mM NaAc, pH5.0, 1M NaCl, resulted in a 95% pure K3 eluting between 300-500mM NaCl. *N*-glycan analysis of the K3 derived glycans is shown in **Fig. 6F**. The earlier removed aliquot of the supernatant was further tested for the presence of secreted mannosidase activity. A commercially available standard of 2-aminobenzamide-labeled N-linked-type oligomannose 9 (Man9-2-AB) (Glyko, Novato, CA) was added to: BMMY (**Fig. 7A**), the supernatant from the above aliquot (**Fig. 7B**), and BMMY containing 10ng of 75mU/mL of α-1,2-mannosidase from *Trichoderma reesei* (obtained from Contreras et al., WO 02/00856 A2) (**Fig. 7C**). After incubation for 24 hours at room temperature, samples were analyzed by amino silica HPLC to determine the extent of mannosidase trimming.

[0270] *P.pastoris* cells comprising plasmid **pGC5** (*Saccharomyces MNS1*(m)/mouse mannosidase IB Δ99) were similarly grown and assayed. Cells were grown at room temperature in BMGY to an OD600 of about 10. Cells were harvested by centrifugation and transferred to BMMY to induce the production of K3 under control of an *AOX1* promoter. After 24 hours of induction, cells were removed by centrifugation to yield an essentially clear supernatant. An aliquot of the supernatant was removed for mannosidase assays and the remainder was used for the recovery of secreted soluble K3. A single purification step using CM-sepharose chromatography and an elution gradient of 25mM NaAc, pH5.0 to 25mM NaAc, pH5.0, 1M NaCl, resulted in a 95% pure K3 eluting between 300-500mM NaCl. *N*-glycan analysis of the K3 derived glycans is shown in **Fig. 5D**. The earlier removed aliquot of the supernatant was further tested for the presence of secreted mannosidase activity as shown in **Fig. 8B**. A commercially available standard of Man9-2-AB (Glyko, Novato, CA) were added to: BMMY (**Fig. 8A**), supernatant from the above aliquot (**Fig. 8B**), and BMMY containing 10ng of 75mU/mL of α-1,2-mannosidase from *Trichoderma reesei* (obtained from Contreras et al., WO 02/00856 A2) (**Fig. 8C**). After incubation for 24 hours at room temperature, samples were analyzed by amino silica HPLC to determine the extent of mannosidase trimming.

[0271] Man9-2-AB was used as a substrate and it is evident that after 24 hours of incubation, mannosidase activity was virtually absent in the supernatant of the

pFB8 (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187) strain digest (**Fig. 7B**) and pGC5 (*Saccharomyces MNS1*(m)/mouse mannosidase IB Δ99) strain digest (**Fig. 8B**) whereas the positive control (purified α-1,2-mannosidase from *T. reesei* obtained from Contreras) leads to complete conversion of $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ under the same conditions, as shown in **Fig. 7C and 8C**. This is conclusive data showing *in vivo* mannosidase trimming in *P.pastoris* pGC5 strain; and pFB8 strain, which is distinctly different from what has been reported to date (Contreras et al., WO 02/00856 A2) .

[0272] **Fig. 9** further substantiates localization and activity of the mannosidase enzyme. *P.pastoris* comprising **pBC18-5** (*Saccharomyces VAN1*(s)/ *C.elegans* mannosidase IB Δ80) was grown at room temperature in BMGY to an OD600 of about 10. Cells were harvested by centrifugation and transferred to BMMY to induce the production of K3 under control of an *AOX1* promoter. After 24 hours of induction, cells were removed by centrifugation to yield an essentially clear supernatant. An aliquot of the supernatant was removed for mannosidase assays and the remainder was used for the recovery of secreted soluble K3. A single purification step using CM-sepharose chromatography and an elution gradient 25mM NaAc, pH5.0 to 25mM NaAc, pH5.0, 1M NaCl, resulted in a 95% pure K3 eluting between 300-500mM NaCl. *N*-glycan analysis of the K3 derived glycans is shown in **Fig. 5E**. The earlier removed aliquot of the supernatant was further tested for the presence of secreted mannosidase activity as shown in **Fig. 9B**. A commercially available standard of Man8-2-AB (Glyko, Novato, CA) was added to: BMMY (**Fig. 9A**), supernatant from the above aliquot pBC18-5 (*Saccharomyces VAN1*(s)/ *C. elegans* mannosidase IB Δ80) (**Fig. 9B**), and BMMY containing media from a different fusion construct pDD28-3 (*Saccharomyces MNN10*(m) (from SwissProt 50108)/*H. sapiens* mannosidase IB Δ99) (**Fig. 9C**). After incubation for 24 hours at room temperature, samples were analyzed by amino silica HPLC to determine the extent of mannosidase trimming. **Fig. 9B** demonstrates intracellular mannosidase activity in comparison to a fusion construct pDD28-3 (*Saccharomyces MNN10*(m) *H. sapiens* mannosidase IB Δ99) exhibiting a negative result (**Fig. 9C**).

Example 14: pH Optimum Assay of Engineered α-1,2-mannosidase

[0273] *P.pastoris* cells comprising plasmid pBB27-2 (*Saccharomyces MNN10* (s) (from SwissProt 50108)/*C. elegans* mannosidase IB Δ31) were grown at room temperature in BMGY to an OD600 of about 17. About 80μL of these cells were inoculated into 600μL BMGY and were grown overnight. Subsequently, cells were harvested by centrifugation and transferred to BMMY to induce the production of K3 (kringle 3 from human plasminogen) under control of an *AOX1* promoter. After 24 hours of induction, cells were removed by centrifugation to yield an essentially clear supernatant (pH 6.43). The supernatant was removed for mannosidase pH optimum assays. Fluorescence-labeled $Man_8GlcNAc_2$ (0.5 μg) was added to 20μL of supernatant adjusted to various pH (**Fig. 11**) and incubated for 8 hours at room temperature. Following incubation the sample was analyzed by HPLC using an Econosil NH2 4.6 X 250 mm, 5 micron bead, amino-bound silica column (Altech, Avondale, PA). The flow rate was 1.0 ml/min for 40 min and the column was maintained to 30°C. After eluting isocratically (68% A:32% B) for 3 min, a linear solvent gradient (68% A:32% B to 40% A:60% B) was employed over 27 min to elute the glycans (18). Solvent A (acetonitrile) and solvent B (ammonium formate, 50 mM, pH 4.5. The column was equilibrated with solvent (68% A:32% B) for 20 min between runs.

Example 15: Engineering of *P.pastoris* to Produce *N*-glycans with the Structure $GlcNAcMan_5GlcNAc_2$

[0274] GlcNAc Transferase I activity is required for the maturation of complex and hybrid *N*-glycans (U.S. Pat. No. 5,834,251). $Man_5GlcNAc_2$ may only be trimmed by mannosidase II, a necessary step in the formation of human glycoforms, after the addition of *N*-acetylglucosamine to the terminal α-1,3 mannose residue of the trimannose stem by GlcNAc Transferase I (Schachter, 1991 Glycobiology 1(5):453-461). Accordingly, a combinatorial DNA library was prepared including DNA fragments encoding suitably targeted catalytic domains of GlcNAc Transferase I genes from *C. elegans* and *Homo sapiens*; and localization

sequences from *GLS, MNS, SEC, MNN9, VAN1, ANP1, HOC1, MNN10, MNN11, MNT1, KTR1, KTR2, MNN2, MNN5, YUR1, MNN1*, and *MNN6* from *S.cerevisiae* and *P.pastoris* putative α-1,2-mannosyltransferases based on the homology from *S.cerevisiae*: D2, D9 and J3, which are *KTR* homologs. **Table 10** includes but does not limit targeting peptide sequences such as *SEC* and *OCH1*, from *P.pastoris* and *K.lactis* GnTI, (See **Table 6 and Table 10**)

Table 10. A representative combinatorial library of targeting peptide sequences/ catalytic domain for UDP-*N*-Acetylglucosaminyl Transferase I (GnTI)

		Targeting peptide				
		***OCHI*(s)**	***OCHI*(m)**	***OCHI*(l)**	***MNN9*(s)**	***MNN9*(m)**
Catalytic Domain	**Human, GnTI, Δ38**	PB105	PB106	PB107	PB104	N/A
	Human, GnTI, Δ86	NB12	NB13	NB14	NB15	NB
	C.*elegans*, GnTI, Δ88	OA12	OA13	OA14	OA15	OA16
	C.*elegans*, GnTI, Δ35	PA12	PA13	PA14	PA15	PA16
	C.*elegans*, GnTI, Δ63	PB12	PB13	PB14	PB15	PB16
	X.*leavis*, GnTI, Δ33	QA12	QA13	QA14	QA15	QA16
	X.*leavis*, GnTI, Δ103	QB12	QB13	QB14	QB15	QB 16

[0275] Targeting peptide sequences were selected from *OCHI* in *P.pastoris* (long, medium and short) (see **Example 11**) and *MNN9* (SwissProt P39107) in *S.cerevisiae* short, and medium. Catalytic domains were selected from human GnTI with a 38 and 86 amino acid *N*-terminal deletion, *C. elegans* (gly-12) GnTI with a 35 and 63 amino acid deletion as well as *C. elegans* (gly-14) GnTI with a 88 amino acid *N*-terminal deletion and *X. leavis* GnTI with a 33 and 103 amino acid *N*-terminal deletion, respectively.

[0276] A portion of the gene encoding human *N*-acetylglucosaminyl Transferase I (MGATI, Accession# NM002406), lacking the first 154 bp, was amplified by PCR using oligonucleotides 5'-TGGCAGGCGCGCCTCAGTCAGCGCTCTCG-3' (SEQ ID NO:32) and 5'-AGGTTAATTA AGTGCTAATTCCAGCTAGG-3' (SEQ ID NO:33) and vector pHG4.5 (ATCC# 79003) as template. The resulting PCR product was cloned into pCR2.1-TOPO and the correct sequence was confirmed. Following digestion with *AscI* and *PacI* the truncated GnTI was inserted into plasmid pJN346 to create pNA. After digestion of pJN271 with *NotI*

and *AscI*, the 120 bp insert was ligated into pNA to generate an in-frame fusion of the *MNN9* transmembrane domain with the GnTI, creating pNA15.

[0277] The host organism is a strain of *P.pastoris* that is deficient in hypermannosylation (e.g. an *och1* mutant), provides the substrate UDP-GlcNAc in the Golgi and/or ER (i.e. contains a functional UDP-GlcNAc transporter), and provides *N*-glycans of the structure $Man_5GlcNAc_2$ in the Golgi and/or ER (e.g. *P.pastoris* pFB8 (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187) from above). First, *P.pastoris* pFB8 was transformed with pPB103 containing the *Kluyveromyces lactis MNN2-2* gene (Genbank AN AF106080) (encoding UDP-GlcNAc transporter) cloned into *BamHI* and *BglII* site of pBLADE-SX plasmid (Cereghino et al. Gene 263 (2001) 159-169). Then the aforementioned combinatorial DNA library encoding a combination of exogenous or endogenous GnTI/localization genes was transformed and colonies were selected and analyzed for the presence of the GnTI construct by colony PCR. Our transformation and integration efficiency was generally above 80% and PCR screening can be omitted once robust transformation parameters have been established.

Protein Purification

[0278] K3 was purified from the medium by Ni-affinity chromatography utilizing a 96-well format on a Beckman BioMek 2000 laboratory robot. The robotic purification is an adaptation of the protocol provided by Novagen for their HisBind resin. Another screening method may be performed using a specific terminal GlcNAc binding antibody, or a lectin such as the GSII lectin from *Griffonia simplificolia*, which binds terminal GlcNAc (EY Laboratories, San Mateo, CA). These screens can be automated by using lectins or antibodies that have been modified with fluorescent labels such as FITC or analyzed by MALDI-TOF.

[0279] Secreted K3 can be purified by Ni-affinity chromatography, quantified and equal amounts of protein can be bound to a high protein binding 96-well plate. After blocking with BSA, plates can be probed with a GSII-FACS lectin and screened for maximum fluorescent response. A preferred method of detecting the above glycosylated proteins involves the screening by MALDI-TOF mass

spectrometry following the affinity purification of secreted K3 from the supernatant of 96-well cultured transformants. Transformed colonies were picked and grown to an OD600 of 10 in a 2ml, 96-well plate in BMGY at 30°C. Cells were harvested by centrifugation, washed in BMMY and resuspended in 250ul of BMMY. Following 24 hours of induction, cells were removed by centrifugation, the supernatant was recovered and K3 was purified from the supernatant by Ni affinity chromatography. The *N*-glycans were released and analyzed by MALDI-TOF delayed extraction mass spectrometry as described herein.

[0280] In summary, the methods of the invention yield strains of *P.pastoris* that produce $GlcNAcMan_5GlcNAc_2$ in high yield, as shown in **Fig. 10B**. At least 60% of the *N*-glycans are $GlcNAcMan_5GlcNAc_2$. To date, no report exists that describes the formation of $GlcNAcMan_5GlcNAc_2$ on secreted soluble glycoproteins in any yeast. Results presented herein show that addition of the UDP-GlcNAc transporter along with GnTI activity produces a predominant $GlcNAcMan_5GlcNAc_2$ structure, which is confirmed by the peak at 1457 (m/z) (**Fig. 10B**).

Construction of strain PBP-3:

[0281] The *P.pastoris* strain expressing K3, (Δ*och1*, arg-, ade-, his-) was transformed successively with the following vectors. First, **pFB8** (*Saccharomyces SEC12* (m)/mouse mannosidase IA Δ187) was transformed in the *P.pastoris* strain by electroporation. Second, **pPB103** containing *Kluyveromyces lactis MNN2-2* gene (Genbank AN AF106080) (encoding UDP-GlcNAc transporter) cloned into pBLADE-SX plasmid (Cereghino et al. Gene 263 (2001) 159-169) digested with *Bam*HI and *Bgl*II enzymes was transformed in the *P.pastoris* strain. Third, **pPB104** containing *Saccharomyces MNN9*(s)/human GnTI Δ38 encoding gene cloned as *Not*I-*Pac*I fragment into pJN336 was transformed into the *P.pastoris* strain.

Example 16: Engineering *K.lactis* Cells to Produce *N*-glycans with the Structure $Man_5GlcNAc_2$

Identification and Disruption of the *K.lactis OCH1* gene

[0282] The *OCH1* gene of the budding yeast *S.cerevisiae* encodes a 1,6-mannosyltransferase that is responsible for the first Golgi localized mannose addition to the $Man_8GlcNAc_2$ *N*-glycan structure on secreted proteins (Nakayama et al, *J Biol Chem.*; 268(35):26338-45 (Dec 15, 1993)). This mannose transfer is generally recognized as the key initial step in the fungal specific polymannosylation of *N*-glycan structures (Nakanishi-Shindo et al, 1993; Nakayama et al, 1992; Morin-Ganet et al, *Traffic* 1(1):56-68. (Jan 2000)). Deletion of this gene in *S.cerevisiae* results in a significantly shorter *N*-glycan structure that does not include this typical polymannosylation or a growth defect at elevated temperatures (Nakayama et al, *EMBO J.*;11(7):2511-9 (Jul 1992)).

[0283] The *Och1p* sequence from *S.cerevisiae* was aligned with known homologs from *Candida albicans* (Genbank accession # AAL49987), and *P.pastoris* (B.K. Choi et al. in prep) along with the *Hoc1* proteins of *S.cerevisiae* (Neiman et al, *Genetics*, 145(3):637-45 (Mar 1997) and *K.lactis* (PENDANT EST database) which are related but distinct mannosyltransferases. Regions of high homology that were in common among *Och1p* homologs but distinct from the *Hoc1p* homologs were used to design pairs of degenerate primers that were directed against genomic DNA from the *K.lactis* strain MG1/2 (Bianchi et al, *Current Genetics* 12, 185-192 (1987)). PCR amplification with primers RCD33 (CCAGAAGAATTCAATTYTGYCARTGG) (SEQ ID NO:34) and RCD34 (CAGTGAAAATACCTGGNCCNGTCCA) (SEQ ID NO:35) resulted in a 302 bp product that was cloned and sequenced and the predicted translation was shown to have a high degree of homology to *Och1* proteins (>55% to *S.cerevisiae Och1p*).

[0284] The 302 bp PCR product was used to probe a Southern blot of genomic DNA from *K.lactis* strain (MG1/2) with high stringency (Sambrook et al, 1989). Hybridization was observed in a pattern consistent with a single gene indicating that this 302 bp segment corresponds to a portion of the *K.lactis* genome and *K.lactis* (*KlOCH1*) contains a single copy of the gene. To clone the entire *KlOCH1* gene, the Southern blot was used to map the genomic locus. Accordingly, a 5.2 kb *Bam*HI/*Pst*I fragment was cloned by digesting genomic DNA and ligating those fragments in the range of 5.2 kb into pUC19 (New England Biolabs, Beverly, MA) to create a *K.lactis* subgenomic library. This subgenomic library was

transformed into *E. coli* and several hundred clones were tested by colony PCR using RCD 33/34. The 5.2 kb clone containing the predicted *KlOCH1* gene was sequenced and an open reading frame of 1362 bp encoding a predicted protein that is 46.5% identical to the *S.cerevisiae OCH1* gene. The 5.2 kb sequence was used to make primers for construction of an *och1::KAN*^{R} deletion allele using a PCR overlap method (Davidson et al, *Microbiology*. 148(Pt 8):2607-15. Aug 2002). This deletion allele was transformed into two *K.lactis* strains and G418 resistant colonies selected. These colonies were screened by both PCR and for temperature sensitivity to obtain a strain deleted for the *OCH1* ORF. The results of the experiment show strains which reveal a mutant PCR pattern, which were characterized by analysis of growth at various temperatures and *N*-glycan carbohydrate analysis of secreted and cell wall proteins following PNGase digestion. The *och1* mutation conferred a temperature sensitivity which allowed strains to grow at 30°C but not at 35°C. **Fig. 12A** shows a MALDI-TOF analysis of a wild type *K.lactis* strain producing N-glycans of $Man_8GlcNAc_2$ **[c]** and higher.

Identification, Cloning, and Disruption of the *K.lactis MNN1* gene

[0285] *S.cerevisiae MNN1* is the structural gene for the Golgi α-1,3-mannosyltransferase. The product of *MNN1* is a 762-amino acid type II membrane protein (Yip et al., *Proc Natl Acad Sci U S A*. 91(7):2723-7. (1994)). Both *N*-linked and *O*-linked oligosaccharides isolated from *mnn1* mutants lack α-1,3-mannose linkages (Raschke et al., *J Biol Chem*., 248(13):4660-6. (Jul 10, 1973).

[0286] The *Mnn1p* sequence from *S.cerevisiae* was used to search the *K.lactis* translated genomic sequences (PEDANT). One 405 bp DNA sequence encoding a putative protein fragment of significant similarity to *Mnn1p* was identified. An internal segment of this sequence was subsequently PCR amplified with primers KMN1 (TGCCATCTTTTAGGTCCAGGCCCGTTC) (SEQ ID NO:36) and KMN2 (GATCCCACGACGCATCGTATTTCTTTC), (SEQ ID NO:37) and used to probe a Southern blot of genomic DNA from *K.lactis* strain (MG1/2). Based on the Southern hybridization data a 4.2 Kb *BamHI-PstI* fragment was cloned by generating a size-selected library as described herein. A single clone containing the *K.lactis MNN1* gene was identified by whole colony PCR using primers KMN1

(SEQ ID NO:36) and KMN2 (SEQ ID NO:37) and sequenced. Within this clone a 2241 bp ORF was identified encoding a predicted protein that was 34% identical to the *S.cerevisiae MNN1* gene. Primers were designed for construction of a *mnn1::NAT*^{R} deletion allele using the PCR overlap method (Davidson et al. 2002).

[0287] This disruption allele was transformed into a strain of *K.lactis* by electroporation and Noursethoicin resistant transformants were selected and PCR amplified for homologous insertion of the disruption allele. Strains that reveal a mutant PCR pattern may be subjected to *N*-glycan carbohydrate analysis of a known reporter gene.

[0288] **Fig. 12B** depicts the *N*-glycans from the *K.lactis och1 mnn1* deletion strain observed following PNGase digestion the MALDI-TOF as described herein. The predominant peak at 1908 (m/z) indicated as **[d]** is consistent with the mass of $Man_9GlcNAc_2$.

Example 17: Engineering Plant Cells To Express GlcNAc Transferases or Galactosyltransferases

[0289] GlcNAc transferase IV is required for the addition of β1,4 GlcNAc to the α-1,6 mannose residue and the α-1,3 mannose residues in complex N-glycans in humans. So far GlcNAc transferase IV has not been detected in or isolated from plants. A transgenic plant that is capable of adding human-like N-glycans must therefore be engineered to express GlcNAc transferase IV. Thus, the plant host cell or transgenic plant must also localize an expressed GlcNAc transferase IV to the correct intracellular compartment in the host so that the enzyme can add the β1,4 GlcNAc to the appropriate mannose residues.

[0290] There is some evidence that glycosyltransferases from mammals and plants have similar targeting signals. For example, a full-length rat α-2,6-sialyltransferase has been shown to correctly localize to the trans Golgi network in transgenic arabidopsis though not necessarily active (Wee E et al. *Plant Cell* 1998 Oct;10(10):1759-68). A fusion construct having fifty-two N-terminal amino acids from α-2,6-sialyltransferase fused to a green fluorescent reporter protein (GFP) was also shown to correctly localize to the plant Golgi (Boevink et al. *Plant J* 1998 Aug;15(3):441-7). Two mammalian proteins -- TGN30 and furin -- and AtELP, an

arabidopsis integral membrane protein (Sanderfoot et al. *Proc Natl Acad Sci U S A* 1998 Aug 18;95(17):9920-5), which localize to the trans Golgi network, each contain a tyrosine tetrapeptide motif which targets them to the Golgi, probably by a recycling mechanism via the plasma membrane. Although mammals and plants appear to share some common mechanisms related to protein targeting, exogenous glycosylases may nonetheless not target correctly in a plant cell, however, localization does not necessarily equal enzyme activity. It therefore becomes essential to devise means to correctly target in a plant cell these enzymes and/or other enzymes which participate in forming complex, human-like N-glycans.

[0291] Glycosylation enzymes are integral membrane proteins which reside in the endoplasmic reticulum and Golgi apparatus. The targeting and localization signals are normally contained in the cytoplasmic and/ or transmembrane domains and in some cases are contained in some lumenal amino acids. For example, fifty-two amino acids that make up the transmembrane domain, nine cytoplasmic amino acids and twenty-six lumenal amino acids of α-2,6-sialyltransferase are required to target GFP to the trans Golgi network (Boevink et al. *Plant J* 1998 Aug;15(3):441-7).

[0292] Thus, a library of sequences encoding cellular targeting signal peptides comprising of either just the cytoplasmic and transmembrane domains or the cytoplasmic, transmembrane and lumenal domains of endoplasmic reticulum and Golgi specific proteins is generated, as described in **Example 11**. The targeting peptide sequences maybe chosen from ER and Golgi-resident plant, yeast or animal proteins. A glycosylation related protein, e.g., an enzyme (or catalytic domain thereof) such as a glycosylase or integral membrane enzyme can be fused in-frame to the library of targeting peptide sequences and introduced into plants (**Fig. 13**). Plant targeting peptide sequences may be most efficient in localizing the chimeric enzymes to the ER and Golgi, although targeting peptide sequences from fungi and mammals may also be effective. For example, the N-terminal 77 amino acids from tobacco *N*-acetylglucosaminyl Transferase I have been shown to correctly target a reporter protein to the Golgi (Essl D. et al., *FEBS Lett* 1999 Jun 18;453(1-2):169-73). In one embodiment, one or more N-terminal fragments comprising these 77 amino acids (or subsets of these amino acids) is fused to one

or more fragments comprising a catalytic domain of GlcNAc transferase IV. At least one resulting fusion protein correctly localizes a functional GlcNAc transferase IV to the Golgi apparatus in a plant cell, as evidenced by monitoring the glycosylation state of a reporter glycoprotein resident or introduced into the plant host cell using techniques described herein.

[0293] Another plant enzyme shown to localize to the Golgi is *Arabidopsis* GlcNAc transferase II (Strasser R et al., *Glycoconj J* 1999 Dec;16(12):787-91). Thus, in another embodiment, one or more different fragments of the arabidopsis GlcNAc transferase II targeting peptide are fused to a GlcNAc transferase IV catalytic domain and fusion constructs produced and tested as described above. The plant specific β1,2-xylosyltransferase from *Arabidopsis thaliana* is another protein that localizes to the Golgi and its localization and retention in the Golgi is dependent on its cytoplasmic and transmembrane sequences (Dirnberger et al., *Plant Mol Biol* 2002 Sep;50(2):273-81). Thus, in another embodiment, one or more fragments comprising the cytoplasmic and transmembrane sequences of β1,2-xylosyltransferase are fused to one or more fragments comprising a GlcNAc transferase IV catalytic domain and resulting fusion constructs are transformed into plant cells and tested for their ability to produce a human-like N-glycan and to otherwise modulate glycosylation in the plant host cell.

[0294] Because GlcNAc transferase IV or Galactosyltransferase from one organism may function more efficiently in a specific plant host than one from another organism, fragments comprising GlcNAc transferase IVs (or catalytic domains) from various eukaryotic organisms are fused in-frame to the library of endoplasmic reticulum (ER) and Golgi targeting peptide sequences and are then introduced into plants. The use of a library of nucleic acids encoding enzyme domains isolated or derived from different species increases the chances of efficient glycosylation - in addition to correct localization and glycosylation by GlcNAc transferase IV.

[0295] The methods and combinatorial nucleic acid libraries of the invention may be used to introduce and localize, sequentially or *en masse*, multiple enzymes required to glycosylate proteins in a plant cell with human-like N-glycans. As different plant species may require different growth conditions, protocols for

transformation may vary depending on the species being transformed (Potrykus, "Gene transfer methods for plants and cell cultures." *Ciba Found Symp* 1990;154:198-208; discussion 208-12). The commonly used methods for generating transgenic plants include Agrobacterium mediated transformation, particle bombardment (Sanford, J.C. et al, Biolistic plant transformation. *Physiol. Plant.* 1990, 79: 206-209) and electroporation.

Agrobacterium method

[0296] The catalytic domains of GlcNAc transferase IVs are fused in-frame to multiple different targeting peptide sequences known to target proteins to the ER and Golgi in plants. Each of these fusion constructs is introduced under the control of the ubiquitously expressed promoters like the 35S CaMV, ubiquitin or actin promoters, tissue specific promoters or inducible promoters. A plant specific terminator region is also used. This cassette (Promoter::targeting peptide-GlcNAc transferase IV::terminator) is cloned into a vector suitable for Agrobacterium mediated transformation **(Fig. 13)**. The vector also contains a selectable marker that allows one to select for transformed plants. The common selectable markers used include those resulting in kanamycin, hygromycin and basta resistance. The construct is introduced into Agrobacterium via well-established transformation methods, which are available in the art. An Agrobacterium library of Golgi-targeted GlcNAc transferase IVs is thereby generated.

[0297] Embryonic and meristematic tissue may be transformed and can regenerate transgenic plants. To transform tissue, tissue explants (these could be plumules and radicals from germinated seeds) are first soaked and coated with an Agrobacterium innoculum. They are then cultured on plates containing the innoculum to form an undifferentiated mass of cells termed the callus. Transformed plant cells are selected for by adding to the medium the relevant kanamycin, hygromycin or basta (depending on the selectable marker used on the construct). The transformed plant cells can either be grown in culture and remain undifferentiated or they are treated with shoot regenerating and shoot elongation medium. Explants that differentiate are transferred onto rooting medium to generate transgenic plants. Some plants like *Arabidopsis* can be transformed by

dipping flowers into an Agrobacterium solution. Seeds from the transformed plants are germinated on plates containing the relevant herbicide or antibiotic selection. Transgenic plants are those that grow on the selection media. The transgenic plants are then screened for those with properly glycosylated proteins (i.e., those which have complex, human-like N-glycans) by isolating glycoproteins from plant extracts and analyzing glycoprotein patterns as described elsewhere herein, e.g., by using a specific antibody or lectin. Although the Agrobacterium method is economical and simple, it is limited to certain species of plants. Accordingly, plants that cannot be transformed using Agrobacterium can be transformed by ballistics or electroporation.

Particle bombardment method and electroporation

[0298] Compared to Agrobacterium mediated transformation, these methods have a greater tendency to insert multiple copies of the transgene into the genome. This could result in gene silencing and cosuppression. However, unlike Agrobacterium mediated transformation, these methods are not species limited and are therefore useful when an Agrobacterium method cannot be employed to generate transgenic plants. In the particle bombardment method, cultured plant cells are bombarded with very small tungsten or gold particle that have been coated with DNA (Promoter::targeting peptide-GlcNAc transferase IV-terminator::selectable marker) (**Fig. 13**) (rb and lb not required) while in the electroporation method, plant cells in a DNA (Promoter::targeting peptide-GlcNAc transferase IV-terminator::selectable marker) solution are treated with an electric pulse that perforates the cell, allowing it to take up DNA. The cells are then cultured and allowed to recover. Stable transformants are selected for by culturing and regenerating plants on appropriate selection medium.

Engineering Soybeans to express GlcNAc transferase IV using a soybean cotyledonary node Agrobacterium mediated transformation system

[0299] An Agrobacterium library of Golgi-targeted GlcNAc transferase IV is generated as described above. Soybean explants are transformed with the library using a protocol described by Hinchee et al (*Bio/Technology* 1988. 6:915). A

reporter protein is expressed with a His tag, purified and then analyzed. Transgenic plants are assayed for proteins with the α-1,6 mannose and the α-1,3 mannose residues using, e.g., mass spectroscopy.

Engineering pea to express GlcNAc transferase IV using particle bombardment

[0300] A GlcNAc transferase IV plasmid library is coated onto tungsten or gold particles and used as microprojectiles to bombard calli derived from pea embryonic tissue as described (Molnar et al., *Symposium on Recent Advances in Plant Biotechnology*, September 4-11, 1999, Stara Lesna, Slovak Republic). A reporter protein is expressed with a His tag, purified and then analyzed. Transgenic plants are assayed for proteins with the α-1,6 mannose and the α-1,3 mannose residues using, e.g., MALDI.

Engineering plants to express GlcNAc transferase I

[0301] GlcNAc transferase I is involved in the addition of GlcNAc to the terminal α-1,3 mannose residue to form $Man_5GlcNAc_2$, an essential step in the maturation of complex N-glycans. Although GlcNAc transferase I has been isolated from plants and appears to have the same function as its mammalian homolog, it may not be the most efficient enzyme for glycosylation of mammalian or exogenous proteins and may not be found in every plant species. As the addition of GlcNAc to the terminal α-1,3 mannose residue is a controlling step in the mammalian glycosylation pathway, it is advantageous to have transgenic plants that can carry out this step efficiently. To create transgenic plants that express GlcNAc transferase I that can function efficiently to promote the formation of complex N-glycans, a library of GlcNAc transferase I isolated or derived from various organisms is fused in-frame to multiple plant Golgi targeting peptide sequences according to the methods described herein. The combinatorial library thus created is introduced into a plant cell or organism as described above for GlcNAc transferase IV.

Engineering maize to express GlcNAc transferase I using particle bombardment

[0302] Transgenic maize can be obtained using a protocol similar to the one used to generate peas that express GlcNAc transferase IV. Here the GlcNAc transferase I plasmid library is coated onto tungsten or gold particles and used to bombard calli derived from maize embryonic tissue, e.g., using a protocol specific for the generation of transgenic maize (Gordon-Kamm WJ et al., *Plant Cell* 1990 Jul;2(7):603-618)). Transgenic plants are assayed for proteins having GlcNAc on the terminal α-1,3 mannose residue, e.g., using specific antibodies or by assaying reduced binding of the N-glycans to certain lectins or by using MALDI-TOF.

[0303] Other useful references for using plant host cells according to the invention include: Christou P. *Plant Mol Biol* 1997 Sep;35(1-2):197-203; Chowrira GM et al. *Mol Biotechnol* 1995 Feb;3(1):17-23; Dirnberger et al., *Plant Mol Biol* 2002 Sep;50(2):273-81; Frame BR et al. *Plant Physiol* 2002 May;129(1):13-22; Gomord V et al. *Biochimie* 1999 Jun;81(6):607-18; Laursen CM et al. *Plant Mol Biol* 1994 Jan;24(1):51-61; Orci L et al. *J Cell Biol* 2000 Sep 18;150(6):1263-70; Newell CA. *Mol Biotechnol* 2000 Sep;16(1):53-65; Pawlowski Wpet al. *Mol Biotechnol* 1996 Aug;6(1):17-30; Schroeder HE et al. *Plant Physiol* 1993 Mar;101(3):751-757; Sorokin, AP et al. *Plant Sci.* 2000 Jul 28;156(2):227-233; Strasser R et al. *Glycoconj J* 1999 Dec;16(12):787-91; and Tomes DT et al. *Plant Mol Biol* 1990 Feb;14(2):261-8.

Engineering plant cells to produce β1,4-galactosyltransferases

[0304] β1,4-galactosyltransferase is an important human glycosyltransferase that is absent in plants. Lerouge P et al. *Plant Mol Biol* 1998 Sep;38(1-2):31-48. In mammals, β1,4-galactosyltransferase is localized in the Golgi and is responsible for the transfer of galactose residues to the terminal N-acetylglucosamine of the core $Man_3GlcNAc_2$ of complex N-glycans. In plants, the $Man_3GlcNAc_2$ core contains β1,2-xylose and α1,3-fucose residues and lacks the β1,4-galactose. The xylose and fucose modifications are implicated in allergies and act as antigenic epitopes and are therefore not desirable modifications of therapeutic proteins.

[0305] The galactose modifications carried out by β1,4-galactosyltransferase can be important for the proper functioning of the therapeutic proteins. In mammals, β1,4-galactosyltransferase acts after N-acetylglucosaminyltransferase I and N-

acetylglucosaminyltransferase II and has been shown to initiate branching of the complex N-glycan. Lerouge P et al. *Plant Mol Biol* 1998 Sep;38(1-2):31-48. Palacpac N et al. *Proc Natl Acad Sci U S A* 1999 Apr 13;96(8):4692-7. In tobacco cells, expression of human β1,4-galactosyltransferase has been shown to result in galactosylated N-glycans with reduced fucose and xylose modifications. Bakker H et al. *Proc Natl Acad Sci U S A* 2001 Feb 27;98(5):2899-904 Fujiyama Ket al. *Biochem Biophys Res Commun* 2001 Nov 30;289(2):553-7. Palacpac N et al. *Proc Natl Acad Sci U S A* 1999 Apr 13;96(8):4692-7. In these studies, a 1.2 kb fragment of human β1,4-galactosyltransferase was cloned downstream of the cauliflower mosaic virus promoter (35SCaMV), introduced into the binary vector pGA482, and finally into tobacco cells. Palacpac N et al. *Proc Natl Acad Sci U S A* 1999 Apr 13;96(8):4692-7.

[0306] Tobacco cells were transformed using the agrobacterium method described by Rempel et al. (Rempel, H.C.et al. 1995. *Transgenic Res.* 4(3):199-207.) Transformation of tobacco cells has also been described (An, G 1985. *Plant Physiol.* 79:568-570). Expression of β1,4-galactosyltransferase under the 35SCaMV resulted in ubiquitous expression of the gene in tobacco cells. Tobacco cells expressing human β1,4-galactosyltransferase showed the presence of galactosylated N-glycans. (Palacpac N et al. *Proc Natl Acad Sci U S A* 1999 Apr 13;96(8):4692-7). Bakker et al. showed that crossing tobacco plants expressing human β1,4-galactosyltransferase with plants expressing the heavy and light chain of a mouse antibody resulted in plants in which the antibody showed 30% galactosylation (Bakker H et al. *Proc Natl Acad Sci U S A* 2001 Feb 27;98(5):2899-904).

[0307] A combinatorial DNA library can be constructed to obtain a β1,4-galactosyltransferase line for the addition of galactose residues. The combinatorial DNA library can effectively produce lines which are more efficient in the addition of galactose residues. Once such a line is made it can be easily crossed to lines expressing other glycosylation enzymes and to those expressing therapeutic proteins to produce therapeutic proteins with human-like glycosylation. The final line can then be grown as plants and harvested to extract proteins or can be cultured as plant cells in suspension cultures to produce proteins in bioreactors. By

expressing the therapeutic proteins using the library of signal peptides, it is possible to retain the therapeutic protein within the cells or have them secreted into the medium. Tobacco cells expressing β1,4-galactosyltransferase secrete galactosylated N-glycans (Ryo Misaki et al. *Glycobiology* 2002 Dec 17;10:1093). While horseradish peroxidase isozyme C expressed in tobacco plants expressing β1,4-galactosyltransferase contained xylose and fucose modifications, no xylose or fucose modification could be detected in horseradish peroxidase isozyme C expressed in tobacco cells expressing β1,4-galactosyltransferase (GT6 cells). (Fujiyama Ket al. *Biochem Biophys Res Commun* 2001 Nov 30;289(2):553-7). This indicates that it may be advantageous to express therapeutic proteins in cell lines instead of whole plants.

Engineering plants to produce sialyltransferase

[0308] In mammals, sialyltransferase is a trans golgi enzyme that adds terminal sialic acid residues to glycosylated polypeptides. Thus far, terminal sialic acid residues have not been detected in plants (Wee E et al. *Plant Cell* 1998 Oct;10(10):1759-68). Wee et al. expressed the rat α-2,6-sialyltransferase in transgenic arabidopsis and showed that the enzyme properly localized to the golgi and was functional. Wee et al. demonstrated that membranes isolated from transgenic arabidopsis, when incubated with CMP-^{3}H-sialic acid and asialofetuin acceptor, resulted in the addition of sialic acid residues while membrane isolated from wild-type arabidopsis did not. While expressing the rat α-2,6-sialyltransferase in arabidopsis resulted in a functional enzyme that was able to incorporate sialic acid residues, fusing the mammalian enzymes α-2,3-sialyltransferase and α-2,6-sialyltransferase to a variety of transit peptides using the library approach of the present invention described earlier can result in more efficient sialylation in other plant species. Wee E et al had to isolate membranes and incubate them with CMP-^{3}H-sialic acid and asialofetuin acceptor since arabidopsis does not have CMP-sialic acid or its transporter. In order to overcome this additional step and obtain sialic acid addition in the plant, CMP-sialic acid biosynthetic pathway and the CMP-sialic acid transporter can be co-expressed in transgenic plants expressing α-2,3-sialyltransferase and α-2,6-sialyltransferase. As

an alternative the CMP-sialic acid transporter can be co-expressed α-2,3-sialyltransferase and α-2,6-sialyltransferase in plant cells grown in suspension culture, and CMP-sialic acid or other precursors of CMP-sialic acid supplied in the medium.

Expressing α-2,3-sialyltransferase and α-2,6-sialyltransferase in lemna

[0309] As described in the U.S. Pat. No. 6,040,498, lemna (duckweed) can be transformed using both agrobacterium and ballistic methods. Using protocols described in the patent, lemna will be transformed with a library of golgi targeted α-2,3-sialyltransferase and/or α-2,6-sialyltransferase and a library of mammalian CMP-sialic acid transporters. Transgenic plants can be assayed for proteins with terminal sialic acid residues.

Expressing α-2,3-sialyltransferase and α-2,6-sialyltransferase in tobacco cells

[0310] Alpha-2,3-sialyltransferase and/or α-2,6-sialyltransferase and/or a library of mammalian CMP-sialic acid transporters can also be introduced into tobacco cells grown in suspension culture as described for β1,4-galactosyltransferases. CMP-sialic acid can be added to the medium. Both the cells and the culture medium (secreted proteins) can be assayed for proteins with terminal sialic acid residues residues.

Example 18: Engineering insect cells to produce glycosyltransferases

[0311] Insect cells provide another mechanism for producing glycoproteins but the resulting glycoproteins are not complex human-like glycoproteins. Marz et al. 1995 *Glycoproteins*, 29:543-563; Jarvis 1997 *The Baculoviruses* 389-431. It is another feature of the present invention to provide enzymes in insect cells, which are targeted to the organelles in the secretory pathway. In a preferred embodiment, enzymes such as glycosyltransferases, galactosyltransferases and sialyltransferases are targeted to the ER, Golgi or the trans Golgi network in lepidopteran insect cells (Sf9). Expression of mammalian β1,4-galactosyltransferase has been shown in Sf9 cells. Hollister et al. *Glycobiology*. 1998 8(5):473-480. These enzymes are targeted by means of a chimeric protein comprising a cellular targeting signal peptide not normally associated with the enzyme. The chimeric proteins are made by constructing a nucleic acid library comprising targeting sequences as described

herein and the glycosylation enzymes. Baculovirus expression in insect cells is commonly used for stable transformation for adding mammalian glycosyltransferases in insect cells. Hollister et al. *Glycobiology*. 2001 11(1):1-9.

Table 11: DNA and Protein Sequence Resources

1. European Bioinformatics Institute (EBI) is a centre for research and services in bioinformatics: http://www.ebi.ac.uk/
2. Swissprot database: http://www.expasy.ch/spr
3. List of known glycosyltransferases and their origin.
4. human cDNA, Kumar et al (1990) Proc. Natl. Acad. Sci. USA 87:9948-9952
5. human gene, Hull et al (1991) Biochem. Biophys. Res. Commun. 176:608-615
6. mouse cDNA, Kumar et al (1992) Glycobiology 2:383-393
7. mouse gene, Pownall et al (1992) Genomics 12:699-704
8. murine gene (5' flanking, non-coding), Yang et al (1994) Glycobiology 5:703-712
9. rabbit cDNA, Sarkar et al (1991) Proc. Natl. Acad. Sci. USA 88:234-238
10. rat cDNA, Fukada et al (1994) Biosci.Biotechnol.Biochem. 58:200-201

1,2 (GnTII) EC 2.4.1.143

11. human gene, Tan et al (1995) Eur. J. Biochem. 231:317-328
12. rat cDNA, D'Agostaro et al (1995) J. Biol. Chem. 270:15211-15221

β1,4 (GnTIII) EC 2.4.1.144

14. human cDNA, Ihara et al (1993) J. Biochem.113:692-698
15. murine gene, Bhaumik et al (1995) Gene 164:295-300
16. rat cDNA, Nishikawa et al (1992) J. Biol. Chem. 267:18199-18204

β1,4 (GnTIV) EC 2.4.1.145

17. human cDNA, Yoshida et al (1998) Glycoconjugate Journal 15:1115-1123
18. bovine cDNA, Minowa et al., European Patent EP 0 905 232

β1,6 (GnT V) EC 2.4.1.155

19. human cDNA, Saito et al (1994) Biochem. Biophys. Res. Commun. 198:318-327
20. rat cDNA, Shoreibah et al (1993) J. Biol. Chem. 268:15381-15385 β1,4 Galactosyltransferase, EC 2.4.1.90 (LacNAc synthetase) EC 2.4.1.22 (lactose synthetase)
21. bovine cDNA, D'Agostaro et al (1989) Eur. J. Biochem. 183:211-217
22. bovine cDNA (partial), Narimatsu et al (1986) Proc. Natl. Acad. Sci. USA 83:4720-4724
23. bovine cDNA (partial), Masibay & Qasba (1989) Proc. Natl. Acad. Sci. USA 86:5733-5377
24. bovine cDNA (5' end), Russo et al (1990) J. Biol. Chem. 265:3324
25. chicken cDNA (partial), Ghosh et al (1992) Biochem. Biophys. Res. Commun. 1215-1222
26. human cDNA, Masri et al (1988) Biochem. Biophys. Res. Commun. 157:657-663

27. human cDNA, (HeLa cells) Watzele & Berger (1990) Nucl. Acids Res. 18:7174
28. human cDNA, (partial) Uejima et al (1992) Cancer Res. 52:6158-6163
29. human cDNA, (carcinoma) Appert et al (1986) Biochem. Biophys. Res. Commun. 139:163-168
30. human gene, Mengle-Gaw et al (1991) Biochem. Biophys. Res. Commun. 176:1269-1276
31. murine cDNA, Nakazawa et al (1988) J. Biochem. 104:165-168
32. murine cDNA, Shaper et al (1988) J. Biol. Chem. 263:10420-10428
33. murine cDNA (novel), Uehara & Muramatsu unpublished
34. murine gene, Hollis et al (1989) Biochem. Biophys. Res. Commun. 162:1069-1075
35. rat protein (partial), Bendiak et al (1993) Eur. J. Biochem. 216:405-417

2,3-Sialyltransferase, (ST3Gal II) (*N*-linked) (Gal-1,3/4-GlcNAc) EC 2.4.99.6

36. human cDNA, Kitagawa & Paulson (1993) Biochem. Biophys. Res. Commun. 194:375-382
37. rat cDNA, Wen et al (1992) J. Biol. Chem. 267:21011-21019

2,6-Sialyltransferase, (ST6Gal I) EC 2.4.99.1

38. chicken, Kurosawa et al (1994) Eur. J. Biochem 219:375-381
39. human cDNA (partial), Lance et al (1989) Biochem. Biophys. Res. Commun. 164:225- 232
40. human cDNA, Grundmann et al (1990) Nucl. Acids Res. 18:667
41. human cDNA, Zettlmeisl et al (1992) Patent EPO475354-A/3
42. human cDNA, Stamenkovic et al (1990) J. Exp. Med. 172:641-643 (CD75)
43. human cDNA, Bast et al (1992) J. Cell Biol. 116:423-435
44. human gene (partial), Wang et al (1993) J. Biol. Chem. 268:4355-4361
45. human gene (5' flank), Aasheim et al (1993) Eur. J. Biochem. 213:467-475
46. human gene (promoter), Aas-Eng et al (1995) Biochim. Biophys. Acta 1261:166-169
47. mouse cDNA, Hamamoto et al (1993) Bioorg. Med. Chem. 1:141-145
48. rat cDNA, Weinstein et al (1987) J. Biol. Chem. 262:17735-17743
49. rat cDNA (transcript fragments), Wang et al (1991) Glycobiology 1:25-31, Wang et al (1990) J. Biol. Chem. 265:17849-17853
50. rat cDNA (5' end), O'Hanlon et al (1989) J. Biol. Chem. 264:17389-17394; Wang et al (1991) Glycobiology 1:25-31
51. rat gene (promoter), Svensson et al (1990) J. Biol. Chem. 265:20863-20688
52. rat mRNA (fragments), Wen et al (1992) J. Biol. Chem. 267:2512-2518

[0312] Additional methods and reagents which can be used in the methods for modifying the glycosylation are described in the literature, such as U.S. Patent No. 5,955,422, U.S. Patent No. 4,775,622, U.S. Patent No. 6,017,743, U.S. Patent No. 4,925,796, U.S. Patent No. 5,766,910, U.S. Patent No. 5,834,251, U.S. Patent No. 5,910,570, U.S. Patent No. 5,849,904, U.S. Patent No. 5,955,347, U.S. Patent No. 5,962,294, U.S. Patent No. 5,135,854, U.S. Patent No. 4,935,349, U.S. Patent No. 5,707,828, and U.S. Patent No. 5,047,335. Appropriate yeast expression systems

can be obtained from sources such as the American Type Culture Collection, Rockville, MD. Vectors are commercially available from a variety of sources.

[0313] SEQUENCE LISTINGS

SEQ ID NO:1-6 can be found in U.S. Pat. Application No. 09/892,591

SEQ ID NO:7
Primer: regions of high homology within 1,6 mannosyltransferases
5'-atggcgaaggcagatggcagt-3'

SEQ ID NO:8
Primer: regions of high homology within 1,6 mannosyltransferases
5'-ttagtccttccaacttccttc-3'

SEQ ID NO:9
internal primer: 5'-actgccatctgccttcgccat-3'

SEQ ID NO:10
internal primer: 5'-GTAATACGACTCACTATAGGGC-3' T7

SEQ ID NO:11
Internal primer: 5'-AATTAACCCTCACTAAAGGG-3' T3

SEQ ID NO:12
Primer: atgcccgtgg ggggcctgtt gccgctcttc agtagc

SEQ ID NO:13
Primer: tcatttctct ttgccatcaa tttccttctt ctgttcacgg

SEQ ID NO:14
Primer: **ggcgcgcc**ga ctcctccaag ctgctcagcg gggtcctgtt ccac

SEQ ID NO:15
Primer: cct**taattaa** tcatttctct ttgccatcaa tttccttctt ctgttcacgg

SEQ ID NO:16
Primer: ggcgagctcg gcctacccgg ccaaggctga gatcatttgt ccagcttcaga

SEQ ID NO:17
Primer: gcccacgtcg acggatccgt ttaaacatcg attggagagg ctgacaccgc tacta

SEQ ID NO:18
Primer: cgggatccac tagtatttaa atcatatgtg cgagtgtaca actcttccca catgg

SEQ ID NO:19
Primer: ggacgcgtcg acggcctacc cggccgtacg aggaatttct cggatgactc ttttc

SEQ ID NO:20
Primer: cgggatccct cgagagatct tttttgtaga aatgtcttgg tgcct

SEQ ID NO:21
Primer: ggacatgcat gcactagtgc ggccgccacg tgatagttgt tcaattgatt gaaataggga caa

SEQ ID NO:22
Primer: ccttgctagc ttaattaacc gcggcacgtc cgacggcggc ccacgggtcc ca

SEQ ID NO:23
Primer: ggacatgcat gcggatccct taagagccgg cagcttgcaa attaaagcct tcgagcgtcc c

SEQ ID NO:24
Primer: gaaccacgtc gacggccatt gcggccaaaa ccttttttcc tattcaaaca caaggcattg c

SEQ ID NO:25
Primer: ctccaatact agtcgaagat tatcttctac ggtgcctgga ctc

SEQ ID NO:26
Primer: tggaaggttt aaacaaagct agagtaaaa tagatatagc gagattagag aatg

SEQ ID NO:27
Primer: aagaattcgg ctggaaggcc ttgtaccttg atgtagttcc cgttttcatc

SEQ ID NO:28
Primer: gcccaagccg gccttaaggg atctcctgat gactgactca ctgataataa aaatacgg

SEQ ID NO:29
Primer: gggcgcgta tttaaatacta gtggatctat cgaatctaaa tgtaagttaa aatctctaa

SEQ ID NO:30
Primer: ggccgcctgc agatttaaat gaattcgg cgcgccttaat

SEQ ID NO:31
Primer: taaggcgcgc cgaattcatt taaatctgca gggc

SEQ ID NO:32
Primer: 5'-tggcaggcgcgcctcagtcagcgctctcg-3'

SEQ ID NO:33
Primer: 5'-aggttaatta agtgctaattccagctagg-3'

SEQ ID NO:34
primer for *K.lactis OCH1* gene: ccagaagaat tcaattytgy cartgg

SEQ ID NO:35
primer for *K.lactis OCH1* gene: cagtgaaaat acctggnccn gtcca

SEQ ID NO:36
primer for *K.lactis MNN1* gene: tgccatcttt taggtccagg cccgttc

SEQ ID NO:37
primer for *K.lactis MNN1* gene: gatcccacga cgcatcgtat ttctttc

SEQ ID NO:38
DNA sequence of the 302 bp segment of the putative *KlOCH1* gene:
gcccttcagtgaaaatacctggcccggtccagttcataatatcggtaccatctgtatttttggcggttttcttttgttgatgttt
gtaatttttgttgaacttctttttatccctcatgttgacattataatcatctgcaatgtcttttaatacttcagc
atcatctaaaggaatgctgcttttaacatttgccacgctctccaatgttgttgcggtgatatttgtgatcaattcgcgcaataa
tggatggccagattttgattgtattgtccactgacaaaattgaattctctggaagggc

SEQ ID NO:39
Translation of putative *KlOCH1* gene (excluding primers):
TIQSKSGHPLLRELITNITATTLESVANVKSSIPLDDAEVLKDIADDYNVNM
RDKKKFNKNYKHQQKKTAKNTDGTDIMN

SEQ ID NO:40
DNA sequence of the 405 bp segment of the putative *KlMNN1* gene:

cccagcgtgccattaccgtatttgccgccgtttgaaatactcaatattcatgatggttgtaaggcgtttttttatcattcgcgat
ataatatgccatctttaggtccaggcccgttctcttagctatctttggtgtctgtgctaccgtgatatggtacct
attctttttccagtctaatctgaagatggcagatttgaaaaaggtagcaacttcaaggtatctttcacaagaaccgtcgttat
cagaacttatgtcaaatgtgaagatcaagcctattgaagaaaccccggtttcgccattggagttgattccagatatcgaaa
tatcgactagaaagaaatacgatgcgtcgtgggatctgttgttccgtggtagaaaatataaatcgttcaacgattatgat

SEQ ID NO:41
DNA sequence of the *K.lactis OCH1* gene:

atggggttaccaaagatttcaagaagaacgaggtacattattgtcattgtgctgatactgtacttattgttttctgtgcaatg
gaatactgcgaaagtgaatcaccatttctataacagcattggcacggtgcttcccagtacagctcgcgtggatcacttga
acttgaaaaacttggacttagcaggtacgagcaataacggtgatcatttgatggatctacgagttcaattggctagtcaat
tcccctacgattctcgagtacccatccccaaaaaggtatggcagacctggaagattgatcccagttcaaagtcacaggtt
tcttccatttcaaaatgccagaatgattggaaacatttcagtgcatccgaggaaccgccatatcaataccaattaatcaca
gatgatcaaatgataccacttctagagcagctatatggtggggtcccacaagtgataaaggcttttgaatccttgccactt
ccaattcttaaagcagacttttcagatacttgatcctttatgcaagaggtggtatatattctgacatggatacgttcccatta
aagccattgtcgtcatggccatcgacttctcagtcctacttttctagtttaaagaatccacaaaggtatagaaattccttgga
caaccttgaaacgctagaagcttcagaacctggctttgtcattggtatcgaggctgatccggatagaagcgattgggca
gagtggtacgccaggagaatacaattctgtcagtggacaatacaatcaaaatctggccatccattattgcgcgaattgat
cacaaatatcaccgcaacaacattggagagcgtggcaaatgttaaaagcagcattcctttagatgatgctgaagtattaa
aagacattgcagatgattataatgtcaacatgagggataaaaagaagttcaacaaaaattacaaacatcaacaaaagaa
aaccgccaaaaatacagatggtaccgatattatgaactggactggtccaggtatttttcagatgttatttccagtatctta
ataacgttatccagaagaatgatgacattttaattttcaatgataatcttaatgttatcaacaaacatggatccaaacatgata
caactatgagattctataaagacattgttaaaaatttacaaaacgacaaaccctcattgttctggggattcttttcattgatga

cagagcctattctagtggacgacatcatggtacttccgattacttctttctcaccaggtatcagaacaatgggcgctaaag
aagacaacgacgagatggcatttgttaagcatatttttgaaggaagttggaaagactga

SEQ ID NO:42
Translation of putative *K.lactis OCH1* gene:

MGLPKISRRTRYIIVIVLILYLLFSVQWNTAKVNHHFYNSIGTVLPSTARVD
HLNLKNLDLAGTSNNGDHLMDLRVQLASQFPYDSRVPIPKKVWQTWKID
PSSKSQVSSISKCQNDWKHFSASEEPPYQYQLITDDQMIPLLEQLYGGVPQ
VIKAFESLPLPILKADFFRYLILYARGGIYSDMDTFPLKPLSSWPSTSQSYFS
SLKNPQRYRNSLDNLETLEASEPGFVIGIEADPDRSDWAEWYARRIQFCQW
TIQSKSGHPLLRELITNITATTLESVANVKSSIPLDDAEVLKDIADDYNVNM
RDKKKFNKNYKHQQKKTAKNTDGTDIMNWTGPGIFSDVIFQYLNNVIQK
NDDILIFNDNLNVINKHGSKHDTTMRFYKDIVKNLQNDKPSLFWGFFSLMT
EPILVDDIMVLPITSFSPGIRTMGAKEDNDEMAFVKHIFEGSWKDZ

SEQ ID NO:43
DNA sequence of the *K.lactis MNN1* gene:

atgatggttgtaaggcgtttttttatcagcttcgcgatataatatgccatcttttaggtccaggcccgttctcttagctatctttg
gtgtctgtgctaccgtgatatggtacctattctttttccagtctaatctgaagatggcagatttgaaaaaggtag
caacttcaaggtatctttcacaagaaccgtcgttatcagaacttatgtcaaatgtgaagatcaagcctattgaagaaaccc
cggtttcgccattggagttgattccagatatcgaaatatcgactagaaagaaatacgatgcgtcgtgggatctgttgttcc
gtggtagaaaatataaatcgttcaacgattatgatcttcatacgaaatgtgagttttatttccagaatttatacaatttgaacg
aggattggaccaataatattcggacgttcactttcgatattaacgatgtagacacgtctacgaaaattgacgctcttaaag
attccgatggggttcaattggtggacgagaaggctatacgtttatacaagagaacgcataacgttgccttggctacgga
aaggttacgtctttatgataaatgttttgtcaatagtccaggttcaaacccattgaaaatggatcacctttcagatcgaaca
agaagagtaagactacggctttggatgacgaagtcactgggaaccgtgatacttttaccaagacgaagaaaacttcgtt
cttaagcgatatggacacgagtagtttccagaagtacgatcaatgggatttcgaacatagaatgttccccatgatcccata
tttcgaggaacacaatttcaccaacgtgatgcctattttcaccggctcaaacggtggggaacctttacctcaagggaaatt
cccggtattagatccaaaatccggtgaattgttacgtgtagagactttcagatatgataaatcgaaatcgctttggaagaa
ctggaatgatatgtcctctgcttctggtaaacgtggtattatcttggctgctggcgacggccaagtggaccaatgcatcc
gtcttattgctacgttgagagctcaaggaaacgctctacctattcaaattatccacaacaaccaattgaatgagaaatctgt
gaaactgttatcggaggccgctaaatctaccgaattctcatccggtagagctcaatctctttggttagtgaatgtgggccc
cacgttggaatcttcaatgaagagcaattttgggagatttaagaataagtggttgtcagttattttcaacacttttgaagaatt
tatattcatagatacagatgccatctcctacattaatatggctgattatttcaacttcaaggagtacaaatctactggaacact
cttctttaaggataggtctttggcaattggaactgaacagaaatgtggtcctttgttcgaaactcttgaaccaagaattcttg
aaatgtactatttcaatactttacctatgatcaatggtgattacgtggaacagcaatgtatgggcatgctcaccccagagg
aaaaagtttacaaacgtttctttgaagttggtcatcaacacaacttggaaagtggattattggccatcaacaaaaacgaac
acatcatgggattggttactgcaacagtcttaaatatcgcaccaaaggtcggaggttgcggttggggtgacaaagagttt
ttctggcttggtttgttggttgctggccaacgctactcgatctatgatatagatgcaagtgcaattggtgttcctcaacagaa
gcaatctatcgctaacggagacgaatttgatgaatataggatttgttctttacaagtggcacatacttcatacgacggacat
ttactatggataaatggtggctctcagtactgtaagaaaccagagacttttgaaggtgattggaccaacattaaggagctt
cgtgaatcgtattctgatgataaagaaaaggctctgaaggcttatagtgatacagttaaggtggaagcagcaatcgtgc
cagattccagaagtaatggttggggtagagacgatcaaagatgtaaaggctacttctggtgcggcaaatttacttcaaa

gctgaaaccgtatacttataacacggtggtaactaaaggtgatttgatccgtttcggagacgaggaaatcgaaagtatct
ccaagattaataagatctggaatgatgctattattccagacggagcttaa

SEQ ID NO:44
Translation of putative *K.lactis MNN1* gene:

MMVVRRFLSASRYNMPSFRSRPVLLAIFGVCATVIWYLFFFQSNLKMADL
KKVATSRYLSQEPSLSELMSNVKIKPIEETPVSPLELIPDIEISTRKKYDASW
DLLFRGRKYKSFNDYDLHTKCEFYFQNLYNLNEDWTNNIRTFTFDINDVD
TSTKIDALKDSDGVQLVDEKAIRLYKRTHNVALATERLRLYDKCFVNSPG
SNPLKMDHLFRSNKKSKTTALDDEVTGNRDTFTKTKKTSFLSDMDTSSFQ
KYDQWDFEHRMFPMIPYFEEHNFTNVMPIFTGSNGGEPLPQGKFPVLDPKS
GELLRVETFRYDKSKSLWKNWNDMSSASGKRGIILAAGDGQVDQCIRLIA
TLRAQGNALPIQIIHNNQLNEKSVKLLSEAAKSTEFSSGRAQSLWLVNVGP
TLESSMKSNFGRFKNKWLSVIFNTFEEFIFIDTDAISYINMADYFNFKEYKST
GTLFFKDRSLAIGTEQKCGPLFETLEPRILEMYYFNTLPMINGDYVEQQCM
GMLTPEEKVYKRFFEVGHQHNLESGLLAINKNEHIMGLVTATVLNIAPKV
GGCGWGDKEFFWLGLLVAGQRYSIYDIDASAIGVPQQKQSIANGDEFDEY
RICSLQVAHTSYDGHLLWINGGSQYCKKPETFEGDWTNIKELRESYSDDKE
KALKAYSDTVKVEAAIVPDSRSNGWGRDDQRCKGYFWCGKFTSKLKPYT
YNTVVTKGDLIRFGDEEIESISKINKIWNDAIIPDGA

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What is claimed is:

1. A method for producing a human-like glycoprotein in a non-human eukaryotic host cell that does not display a 1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the step of introducing into the host cell one or more enzymes for production of a $Man_5GlcNAc_2$ carbohydrate structure, wherein $Man_5GlcNAc_2$ is produced within the host cell at a yield of at least 30 mole percent.

2. The method of claim 1, wherein at least 10 percent of the $Man_5GlcNAc_2$ produced within the host cell is a productive substrate for GnTI *in vivo*.

3. The method of claim 1, wherein at least one of the enzymes is selected to have optimal activity at the pH of the location in the host cell where the carbohydrate structure is produced.

4. The method of claim 2, wherein at least one of the enzymes is selected to have a pH optimum within about 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the enzyme is localized.

5. The method of claim 1, wherein at least one of the enzymes is targeted to a subcellular location in the host cell where the enzyme will have optimal activity.

6. The method of claim 4, wherein the enzyme is targeted by means of a chimeric protein comprising a cellular targeting signal peptide not normally associated with the enzyme.

7. The method of claim 1, wherein at least one introduced enzyme is targeted to the endoplasmic reticulum, the early, medial, late Golgi or the trans Golgi network of the host cell.

8. The method of claim 1, wherein at least one of the enzymes is selected from the group consisting of mannosidases, glycosyltransferases and glycosidases.

9. The method of claim 6, wherein the enzyme is a mannosidase predominantly localized in the Golgi apparatus or the endoplasmic reticulum.

10. The method of claim 1, wherein the glycoprotein comprises *N*-glycans of which greater than 30 mole percent comprise six or fewer mannose residues.

11. The method of claim 1, wherein the glycoprotein comprises *N*-glycans of which greater than 30 mole percent comprise three or fewer mannose residues.

12. The method of claim 1, wherein the glycoprotein comprises one or more sugars selected from the group consisting of GlcNAc, galactose, sialic acid, and fucose.

13. The method of claim 1, wherein the glycoprotein comprises at least one oligosaccharide branch comprising the structure NeuNAc-Gal-GlcNAc-Man.

14. The method of claim 1, wherein the host is selected from the group consisting of plant, algae, insect, fungi, yeast cells.

15. The method of claim 1, wherein the host is a lower eukaryotic cell.

16. The method of claim 15, wherein the host cell is selected from the group consisting of *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*,

Chrysosporium lucknowense, *Fusarium* sp., *Fusarium gramineum*, *Fusarium venenatum* and *Neurospora crassa*.

17. The method of claim 1, wherein the host is deficient in the activity of one or more enzymes selected from the group consisting of mannosyltransferases and phosphomannosyltransferases.

18. The method of claim 17, wherein the host does not express an enzyme selected from the group consisting of 1,6 mannosyltransferase; 1,3 mannosyltransferase; and 1,2 mannosyltransferase.

19. The method of claim 1, wherein the host is an *och1* mutant of *P. pastoris*.

20. The method of claim 1, wherein the host expresses GnTI and UDP-GlcNAc transporter activities.

21. The method of claim 1, wherein the host expresses a UDP- or GDP-specific diphosphatase activity.

22. The method of claim 1, further comprising the step of isolating the glycoprotein from the host.

23. The method of claim 22, further comprising the step of subjecting the isolated glycoprotein to at least one further glycosylation reaction *in vitro*, subsequent to its isolation from the host.

24. The method of claim 1, wherein the step of introducing into the host cell one or more enzymes for production of the $Man_5GlcNAc_2$ carbohydrate structure comprises a nucleic acid molecule.

25. The method of claim 1, further comprising the step of introducing into the host a nucleic acid molecule encoding one or more mannosidase activities involved in the production of $Man_5GlcNAc_2$ from $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$.

26. The method of claim 25, wherein at least one of the encoded mannosidase activities has a pH optimum within about 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the mannosidase activity is localized, or has optimal activity at a pH of between about 5.1 and about 8.0.

27. The method of claim 26, wherein the mannosidase has optimal activity at a pH of between about 5.5 and about 7.5.

28. The method of claim 26, wherein the mannosidase activity is an α-1,2-mannosidase derived from mouse, human, *Lepidoptera*, *Aspergillus nidulans*, or *Bacillus* sp., *C.elegans, D.melanogaster, P.citrinum* or *X.laevis.*

29. The method of claim 24, wherein at least one enzyme is localized by forming a fusion protein comprising a catalytic domain of the enzyme and a cellular targeting signal peptide.

30. The method of claim 29, wherein the fusion protein is encoded by at least one genetic construct formed by the in-frame ligation of a DNA fragment encoding a cellular targeting signal peptide with a DNA fragment encoding a catalytic domain having enzymatic activity.

31. The method of claim 30, wherein the encoded targeting signal peptide is derived from a member of the group consisting of: membrane-bound proteins of the ER or Golgi, retrieval signals, Type II membrane proteins, Type I membrane proteins, membrane spanning nucleotide sugar transporters, mannosidases,

sialyltransferases, glucosidases, mannosyltransferases and phospho-mannosyltransferases.

32. The method of claim 24, wherein the catalytic domain encodes a glycosidase, mannosidase or a glycosyltransferase activity derived from a member of the group consisting of GnTI, GnTII, GnTIII, GnTIV, GnT V, GnT VI, GalT, Fucosyltransferase and Sialyltransferase, and wherein the catalytic domain has a pH optimum within 1.4 pH units of the average pH optimum of other representative enzymes in the organelle in which the enzyme is localized, or has optimal activity at a pH between 5.1 and 8.0.

33. The method of claim 32, wherein the catalytic domain encodes a mannosidase selected from the group consisting of *C. elegans* mannosidase IA, *C. elegans* mannosidase IB, *D. melanogaster* mannosidase IA, *H. sapiens* mannosidase IB, *P. citrinum* mannosidase I, mouse mannosidase IA, mouse mannosidase IB, *A. nidulans* mannosidase IA, *A. nidulans* mannosidase IB, *A. nidulans* mannosidase IC, mouse mannosidase II, *C. elegans* mannosidase II, *H. sapiens* mannosidase II, and mannosidase III.

34. The method of claim 24, wherein the nucleic acid molecule encodes one or more enzymes selected from the group consisting of UDP-GlcNAc transferase, UDP-galactosyltransferase, GDP-fucosyltransferase, CMP-sialyltransferase, UDP-GlcNAc transporter, UDP-galactose transporter, GDP-fucose transporter, CMP-sialic acid transporter, and nucleotide diphosphatases.

35. The method of claim 24, wherein the host expresses GnTI and UDP-GlcNAc transporter activities.

36. The method of claim 24, wherein the host expresses a UDP- or GDP-specific diphosphatase activity.

37. A nucleic acid library comprising at least two different genetic constructs, wherein at least one genetic construct comprises a nucleic acid fragment encoding a glycosylation enzyme ligated in-frame with a nucleic acid fragment encoding a cellular targeting signal peptide which it is not normally associated with.

38. A DNA library of fusion constructs comprising:

(a) at least two nucleotide sequences encoding a cellular targeting signal peptide and at least one nucleotide sequence encoding a catalytic domain region selected from the group consisting of mannosidases, glycosyltransferases and glycosidases; or

(b) at least one nucleotide sequence encoding a cellular targeting signal peptide and at least two nucleotide sequences encoding a catalytic domain region selected from the group consisting of mannosidases, glycosyltransferases and glycosidases;

wherein at least one nucleotide sequence encoding a catalytic domain region is ligated in-frame to a nucleotide sequence encoding a cellular targeting signal peptide.

39. The DNA library of claim 37 comprising at least one nucleic acid comprising a naturally occurring sequence encoding a glycosylation enzyme.

40. The DNA library of claim 37 comprising at least one nucleic acid sequence previously subjected to a technique selected from the list: gene shuffling, *in vitro* mutagenesis, and error-prone polymerase chain reaction.

41. The DNA library of claim 37, wherein the glycosyltransferase is selected from the group consisting of: mannosyltransferases, GlcNAc transferases, phospho-GlcNAc transferases, galactosyltransferases, sialyltransferases and fucosyltransferases.

42. The DNA library of claim 37, wherein at least one nucleotide sequence encoding a catalytic domain region is derived from mouse, human, *C.elegans, D.melanogaster, P.citrinum, X.laevis, Bacillus sp.*or *A.nidulans*.

43. The DNA library of claim 37, wherein the mannosidase catalytic domain is selected from the group consisting of: *C. elegans* mannosidase IA, *C. elegans* mannosidase IB, *D. melanogaster* mannosidase IA, *H. sapiens* mannosidase IB, *P. citrinum* mannosidase I, mouse mannosidase IA, mouse mannosidase IB, *A. nidulans* mannosidase IA, *A. nidulans* mannosidase IB, *A. nidulans* mannosidase IC, mouse mannosidase II, *C. elegans* mannosidase II, *H. sapiens* mannosidase II, and mannosidase III.

44. The DNA library of claim 37, wherein the nucleic acid fragment encoding a cellular targeting signal peptide is selected from the group consisting of: membrane-bound proteins of the ER or Golgi, retrieval signals, Type II membrane proteins, Type I membrane proteins, membrane spanning nucleotide sugar transporters, mannosidases, sialyltransferases, glucosidases, mannosyltransferases and phosphomannosyltransferases.

45. The DNA library of claim 37, wherein the nucleic acid fragment encoding a cellular targeting peptide is selected from the group consisting of: *Saccharomyces* GLS1, *Saccharomyces* MNS1, *Saccharomyces* SEC12, *Pichia* SEC, *Pichia* OCH1, *Saccharomyces* MNN9, *Saccharomyces* VAN1, *Saccharomyces* ANP1,

Saccharomyces HOC1, *Saccharomyces* MNN10, *Saccharomyces* MNN11, *Saccharomyces* MNT1, *Pichia* D2, *Pichia* D9, *Pichia* J3, *Saccharomyces* KTR1, *Saccharomyces* KTR2, *Kluyveromyces* GnTI, *Saccharomyces* MNN2, *Saccharomyces* MNN5, *Saccharomyces* YUR1, *Saccharomyces* MNN1, and *Saccharomyces* MNN6.

46. A vector comprising a fusion construct derived from a DNA library of any one of claims 37-45 operably linked to an expression control sequence, wherein said cellular targeting signal peptide is targeted to the ER, Golgi or trans-Golgi network.

47. The vector of claim 46 which, upon expression in a host cell, encodes a mannosidase activity involved in producing $Man_5GlcNAc_2$ *in vivo*.

48. The vector of claim 46 which, upon expression in a host cell, encodes a glycosyltransferase activity involved in producing $GlcNAcMan_5GlcNAc_2$ *in vivo*.

49. A eukaryotic host cell comprising at least one vector of claim 46.

50. The host cell of claim 49, selected from the group consisting of unicellular and multicellular fungi.

51. The host cell of claim 49, selected from the group consisting of *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium*

lucknowense, Fusarium sp. Fusarium gramineum, Fusarium venenatum and *Neurospora crassa.*

52. A method for producing a human-like glycoprotein in a non-human cell comprising the step of culturing a eukaryotic host cell comprising at least one vector of claim 46.

53. The method of claim 52, wherein the host is a unicellular or multicellular fungal cell.

54. The method of claim 52, wherein the host cell is selected from the group consisting of *Pichia pastoris, Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis, Pichia methanolica, Pichia* sp., *Saccharomyces cerevisiae, Saccharomyces* sp., *Hansenula polymorpha, Kluyveromyces* sp., *Kluyveromyces lactis Candida albicans, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense, Fusarium sp. Fusarium gramineum, Fusarium venenatum* and *Neurospora crassa.*

55. A method for producing a human-like glycoprotein in a non-human host cell comprising the step of transforming the host cell with a DNA library of any one of claims 37-45 to produce a genetically mixed cell population expressing at least one glycosylation enzyme derived from the library.

56. The method of claim 55, further comprising the step of selecting from the mixed cell population a cell producing a desired human-like glycosylation phenotype.

57. The method of claim 56, wherein the selection comprises the step of analyzing a glycosylated protein or isolated *N*-glycan by one or more methods selected from the group consisting of: (a) mass spectroscopy; (b) MALDI-TOF; (c) liquid chromatography; (d) characterizing cells using a fluorescence activated cell sorter, spectrophotometer, fluorimeter, or scintillation counter; (e) exposing host cells to a lectin or antibody having a specific affinity for a desired oligosaccharide moiety; and (f) exposing cells to a cytotoxic or radioactive molecule selected from the group consisting of sugars, antibodies and lectins.

58. The method of claim 55, wherein the DNA fragment encoding the catalytic domain has an activity selected from the group consisting of mannosidase, UDP-GlcNAc transferase, UDP-galactosyltransferase, and CMP-sialyltransferase activity and wherein the cellular targeting signal peptide localizes the enzyme predominantly in a host cell organelle selected from the group consisting of endoplasmic reticulum, cis Golgi, medial Golgi, and trans Golgi.

59. The method of claim 55, wherein said host cell further comprises a target glycoprotein of interest on which $Man_5GlcNAc_2$ is produced *in vivo*.

60. The method of claim 59, wherein the $Man_5GlcNAc_2$ produced *in vivo* is the predominant N-glycan on the target glycoprotein.

61. The method of claim 55, wherein said host cell further comprises a target glycoprotein of interest on which $GlcNAcMan_5GlcNAc_2$ is produced *in vivo*.

62. The method of claim 61, wherein the $GlcNAcMan_5GlcNAc_2$ produced *in vivo* is the predominant N-glycan on the target glycoprotein.

63. A host cell produced by the method of claim 1, 24 or 55.

64. A human-like glycoprotein produced by the method of claim 1, 24 or 55.

65. A method for altering the glycosylation pattern of a eukaryotic cell comprising the step of transforming the host cell with a DNA library of any one of claims 37-45 to produce a genetically mixed cell population expressing at least one glycosylation enzyme derived from the library.

66. An isolated nucleic acid molecule comprising or consisting of nucleic acid sequences selected from the group consisting of: (a) at least forty-five (45) contiguous nucleotide residues of SEQ ID NO:41; (b) homologs, variants and derivatives of (a); and (c) nucleic acid sequences that hybridize under stringent conditions to (a) but excluding sequences which encode the *S. cerevisiae OCH1* gene.

67. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO:42.

68. An isolated nucleic acid molecule of claim 66 which encodes an *OCH1* activity upon expression in a host cell.

69. An isolated nucleic acid molecule of claim 66 which encodes a *K.lactis OCH1* gene.

70. An isolated nucleic acid molecule comprising or consisting of nucleic acid sequences selected from the group consisting of: (a) at least forty-five (45) contiguous nucleotide residues of SEQ ID NO:43; (b) homologs, variants and derivatives of (a); and (c) nucleic acid sequences that hybridize under stringent conditions to (a) but excluding sequences which encode the *S. cerevisiae MNN1* gene.

71. An isolated polypeptide comprising the amino acid sequence of SEQ ID NO:44.

72. An isolated nucleic acid molecule of claim 70 which encodes an *MNN1* gene.

73. An isolated nucleic acid molecule of claim 70, wherein said sequence encodes a *K.lactis MNN1* gene.

74. A host cell comprising a disruption or mutation of SEQ ID NO:41 which is characterized by having a reduced expression level of SEQ ID NO:41 compared to a host cell without said disruption or mutation.

75. A host cell comprising a disruption or mutation of SEQ ID NO:43 which is characterized by having a reduced expression level of SEQ ID NO:43 compared to a host cell without said disruption or mutation.

76. A method of modifying plant glycosylation comprising introducing into a host at least one nucleotide sequence encoding a catalytic domain region ligated in-frame to a nucleotide sequence encoding a cellular targeting signal peptide.

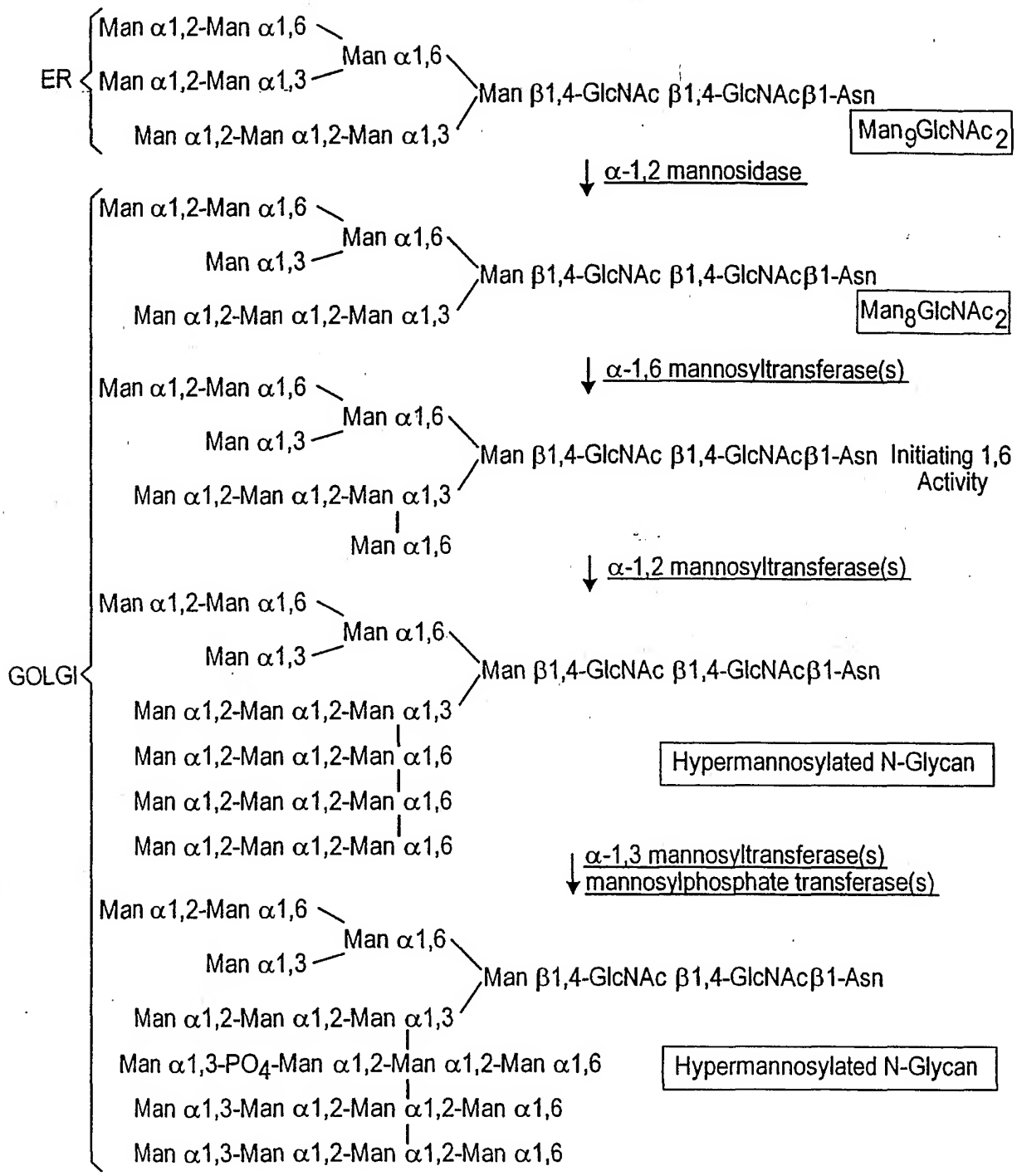

ER
Man α1,2-Man α1,6
Man α1,6
Man α1,2-Man α1,3
Man α1,2-Man α1,2-Man α1,3
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Man9GlcNAc2
α-1,2 mannosidase
Man8GlcNAc2
α-1,6 mannosyltransferase(s)
Initiating 1,6 Activity
α-1,2 mannosyltransferase(s)
GOLGI
Hypermannosylated N-Glycan
α-1,3 mannosyltransferase(s)
mannosylphosphate transferase(s)
Man α1,3-PO4-Man α1,2-Man α1,2-Man α1,6
Man α1,3-Man α1,2-Man α1,2-Man α1,6

FIG. 1A

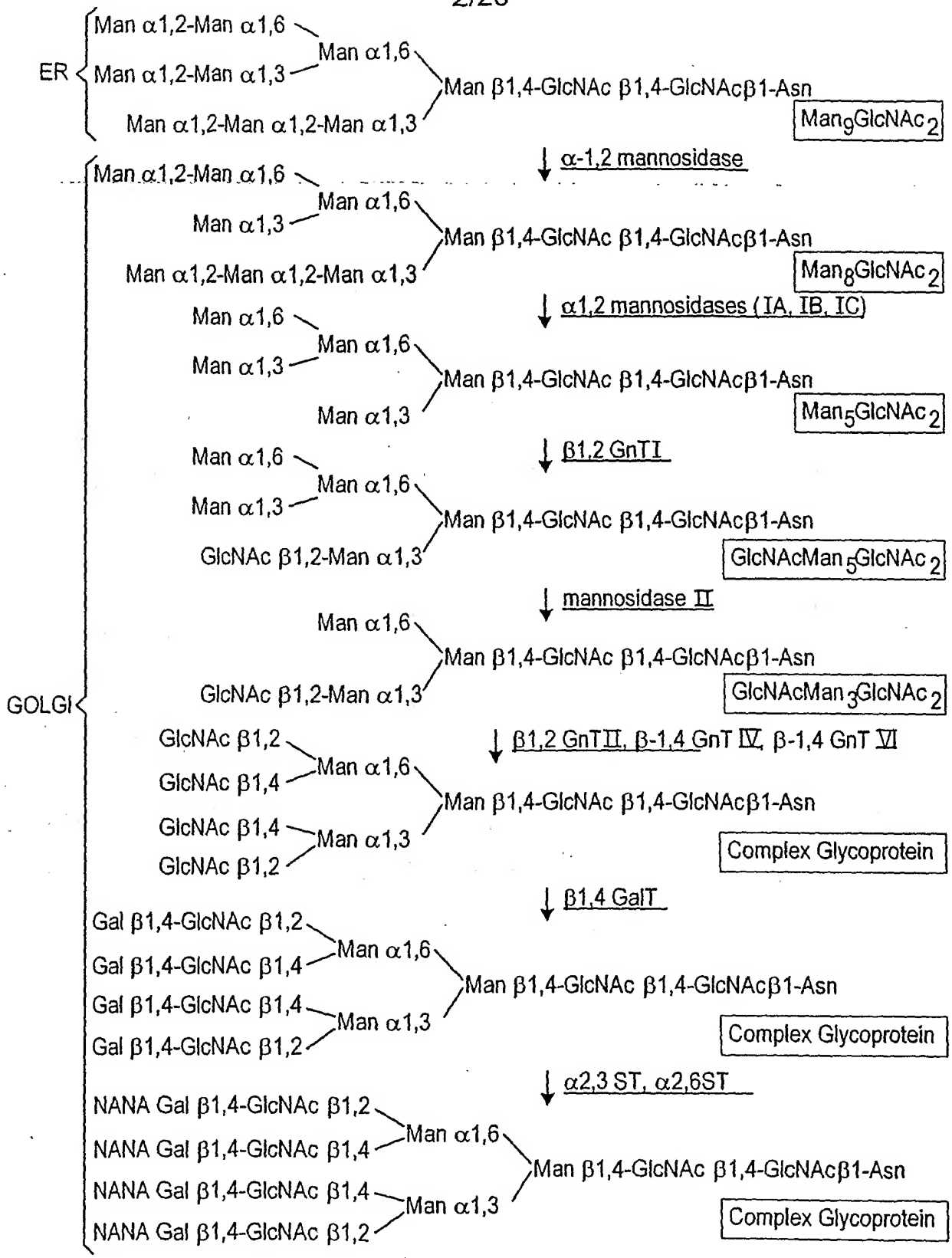

ER
Man α1,2-Man α1,6
Man α1,2-Man α1,3
Man α1,2-Man α1,2-Man α1,3
Man α1,6
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Man9GlcNAc2
α-1,2 mannosidase
Man α1,2-Man α1,6
Man α1,3
Man α1,2-Man α1,2-Man α1,3
Man α1,6
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Man8GlcNAc2
α1,2 mannosidases (IA, IB, IC)
Man α1,6
Man α1,3
Man α1,3
Man α1,6
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Man5GlcNAc2
β1,2 GnTI
Man α1,6
Man α1,3
GlcNAc β1,2-Man α1,3
Man α1,6
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
GlcNAcMan5GlcNAc2
mannosidase II
Man α1,6
GlcNAc β1,2-Man α1,3
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
GlcNAcMan3GlcNAc2
GOLGI
β1,2 GnTII, β-1,4 GnT IV, β-1,4 GnT VI
GlcNAc β1,2
GlcNAc β1,4
GlcNAc β1,4
GlcNAc β1,2
Man α1,6
Man α1,3
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Complex Glycoprotein
β1,4 GalT
Gal β1,4-GlcNAc β1,2
Gal β1,4-GlcNAc β1,4
Gal β1,4-GlcNAc β1,4
Gal β1,4-GlcNAc β1,2
Man α1,6
Man α1,3
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Complex Glycoprotein
α2,3 ST, α2,6ST
NANA Gal β1,4-GlcNAc β1,2
NANA Gal β1,4-GlcNAc β1,4
NANA Gal β1,4-GlcNAc β1,4
NANA Gal β1,4-GlcNAc β1,2
Man α1,6
Man α1,3
Man β1,4-GlcNAc β1,4-GlcNAcβ1-Asn
Complex Glycoprotein

FIG. 1B

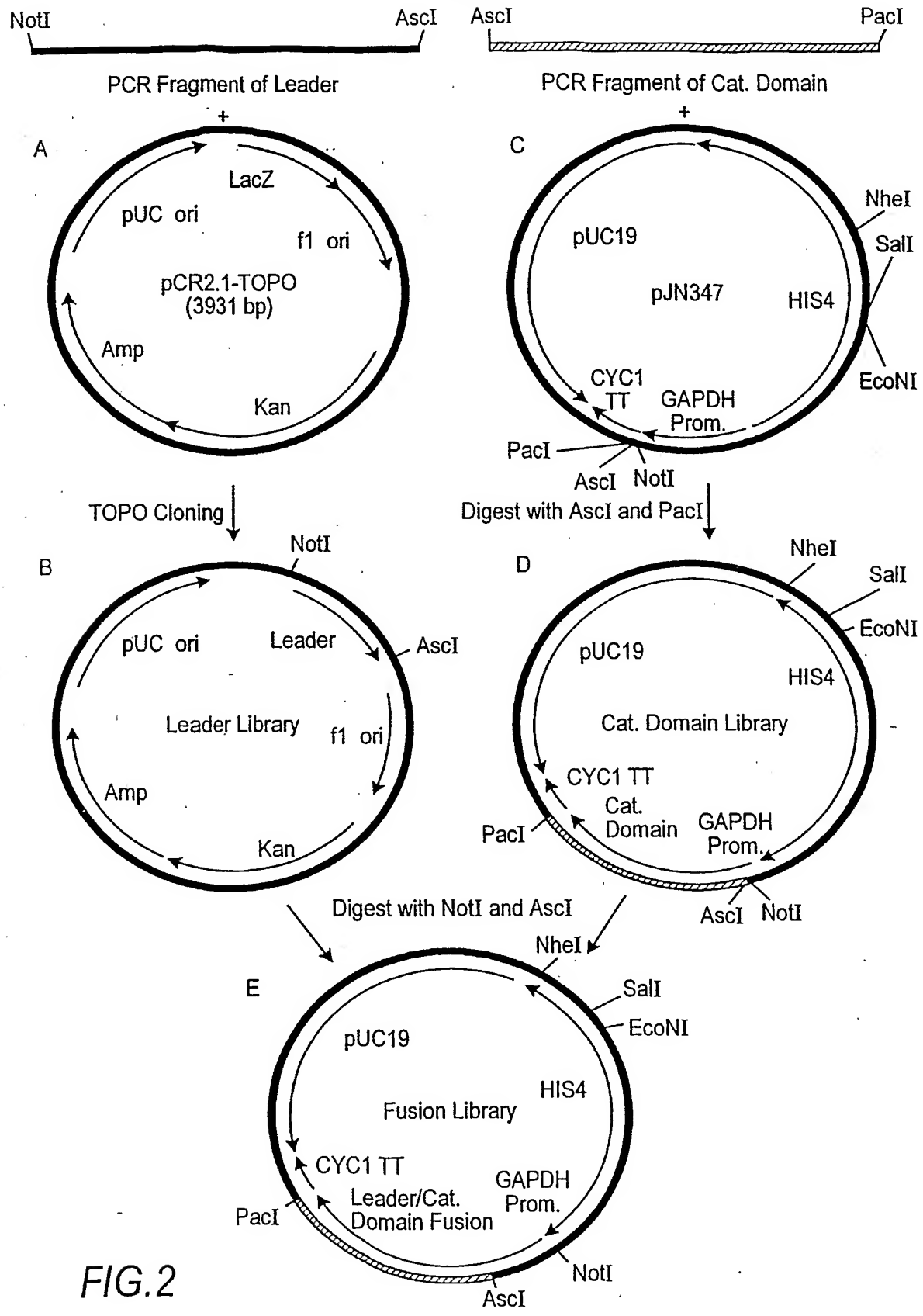

NotI
AscI
AscI
PacI
PCR Fragment of Leader
PCR Fragment of Cat. Domain
A
LacZ
pUC ori
f1 ori
pCR2.1-TOPO
(3931 bp)
Amp
Kan
C
NheI
SalI
pUC19
pJN347
HIS4
EcoNI
CYC1
TT
GAPDH
Prom.
PacI
AscI
NotI
TOPO Cloning
Digest with AscI and PacI
B
NotI
pUC ori
Leader
AscI
Leader Library
f1 ori
Amp
Kan
D
NheI
SalI
EcoNI
pUC19
HIS4
Cat. Domain Library
CYC1 TT
Cat.
Domain
GAPDH
Prom.
PacI
AscI
NotI
Digest with NotI and AscI
NheI
E
SalI
EcoNI
pUC19
HIS4
Fusion Library
CYC1 TT
GAPDH
Prom.
Leader/Cat.
Domain Fusion
PacI
NotI
AscI

FIG.2

M. musculus alpha-1,2-mannosidase IA open reading frame. The transmembrane and catalytic domains are highlighted in bold respectively. The sequence of the primers used to generate the N-terminal truncations are highlighted by underlining and the start of each respective protein fragment indicated by an arrow.

```
   1 atgcccgtgggggcctgttgccgctcttcagtagccctgggggcggcggcctgggcagtggcctgggcgggggcttggcggcgggaggaagggg
   1 M  P  V  G  G  L  L  P  L  F  S  S  P  G  G  G  G  L  G  S  G  L  G  G  G  L  G  G  G  R  K  G
  97 tctggccccgctgccttccgcctcaccgagaagttcgtgctgctgctggtgttcagcgccttcatcacgctctgcttcgggggcaatc
  33 S  G  P  A  A  F  R  L  T  E  K  F  V  L  L  L  V  F  S  A  F  I  T  L  C  F  G  A  I
 184 ttcttcctgcctgactcctccaagctgctcagcggggtcctgttccactccaaccctgccttgcagccgccggcggagcacaagcccgggctcg
  62 F  F  L  P  D  S  S  K  L  L  S  G  V  L  F  H  S  N  P  A  L  Q  P  P  A  E  H  K  P  G  L
                 d65 primer
 278 gggcgcgtgcggaggatgccgccgaggggagagtccggcaccgcgaggaaggcgcgcctggggaccctggagctggactggaagacaacttagcca
  93 G  A  R  A  E  D  A  A  E  G  R  V  R  H  R  E  E  G  A  P  G  D  P  G  A  G  L  E  D  N  L  A
                                 d105 primer
 374 ggatccgcgaaaaccacgagcgggctctcagggaagccaaggagaccctgcagaagctgccggaggagatccaaagagacattctgctggagaagg
 125 R  I  R  E  N  H  E  R  A  L  R  E  A  K  E  T  L  Q  K  L  P  E  E  I  Q  R  D  I  L  L  E  K
 470 aaaaggtggcccaggaccagctgcgtgacaaggatctgtttaggggcttgcccaaggtggacttcctgccccccgtcggggtagagaaccgggagc
 157 E  K  V  A  Q  D  Q  L  R  D  K  D  L  F  R  G  L  P  K  V  D  F  L  P  P  V  G  V  E  N  R  E
                                                                                 d187 primer
 566 ccgctgacgccaccatccgtgagaagagggcaaagatcaaagagatgatgacccatgcttggaataattataaacgctatgcgtggggc
 189 P  A  D  A  T  I  R  E  K  R  A  K  I  K  E  M  M  T  H  A  W  N  N  Y  K  R  Y  A  W  G
```

FIG. 3

```
655 ttgaacgaactgaaacctatatcaaagaaggccattcaagcagtttgtttggcaacatcaaaggagctacaatagtagatg
219▶ L N E L K P I S K E G H S S S L F G N I K G A T I V D
737 ccctggataccctttcattatgggcatgaagactgaatttcaagaagctaaatcgtggattaaaaatatttagatttaa
246▶A L D T L F I M G M K T E F Q E A K S W I K K Y L D F N
819 tgtgaatgctgaagtttctgtttttgaagtcaacatacgcttcgtcggtggactgctgtcagcctactatttgtccggagag
273▶ V N A E V S V F E V N I R F V G G L L S A Y Y L S G E
901 gagatatttcgaaagaaagcagtggaacttgggtaaaattgctacctgcatttcatactccctctggaatacctttgggcat
301▶E I F R K K A V E L G V K L L P A F H T P S G I P W A
983 tgctgaatatgaaaagtgggatcgggcggaactggccctgggcctctgggaggcagcagtatcctggccgaatttggaactct
328▶L L N M K S G I G R N W P W A S G G S S I L A E F G T L
1065 gcatttagagtttatgcacttgtcccacttatcaggagaccagtctttgccgaaaaggttatgaaattcgaacagtgttg
355▶ H L E F M H L S H L S G D P V F A E K V M K I R T V L
1147 aacaaactggacaaaccagaaggcctttatcctaactatctgaaccccagtagtggacagtgggtcaacatcatgtctcgg
383▶N K L D K P E G L Y P N Y L N P S S G Q W G Q H H V S
1229 ttggaggacttggagacagctttttatgaatatatttgctcttaaggcgtggttaatgtctgacaagacagatctcgaagccaagaa
410▶V G G L G D S F Y E Y L L K A W L M S D K T D L E A K K
1311 gatgtatattttgatgctgttcaggccatcgagactcacttgatccgcaagtcaagtgggggactaacgtacatcgcagagtgg
437▶ M Y F D A V Q A I E T H L I R K S S G G L T Y I A E W
1393 aaggggggcctcctggaacacaagatgggccacctgacgtgctttgcaggaggcatgtttgcacttgggggcagatggagctc
465▶K G G L L E H K M G H L T C F A G G M F A L G A D G A
1475 cggaagcccgggcccaacactaccttgaactcggagctgaaattgcccgcacttgtcatgaatcttataatcgtacatatgt
492▶P E A R A Q H Y L E L G A E I A R T C H E S Y N R T Y V
1557 gaagttgggaccggaagcgtttcgatttgatggcggtgtggaagctattgccacgaggcaaaatgaaaagtattacatcttta
519▶ K L G P E A F R F D G G V E A I A T R Q N E K Y Y I L
1639 cggcccgaggtcatcgagacatacatgtacatgtggcgactgactcacgacccaagtacaggacctgggcctgggaagccg
547▶R P E V I E T Y M Y W R L T H D P K Y R T W A W E A
1721 tggaggctctagaaagtcactgcagaggtgaacggaggctactcaggcttacgggatgtttacattgcccgtgagagttatga
574▶V E A L E S H C R V N G G Y S G L R D V Y I A R E S Y D
1803 cgatgtccagcaaagtttcttcctggcagagacactgaagtatttgtacttgatatttccgatgatgaccttcttccaccta
601▶ D V Q Q S F F L A E T L K Y L Y I F S D D D L L P L
1885 gaacactggatcttcaacaccgaggctcatccttccctatacttccgtgaacagaagaaggaaattgatggcaaagagaaatga
629▶E H W I F N T E A H P F P I L R E Q K K E I D G K E K
```

FIG. 3 CONT

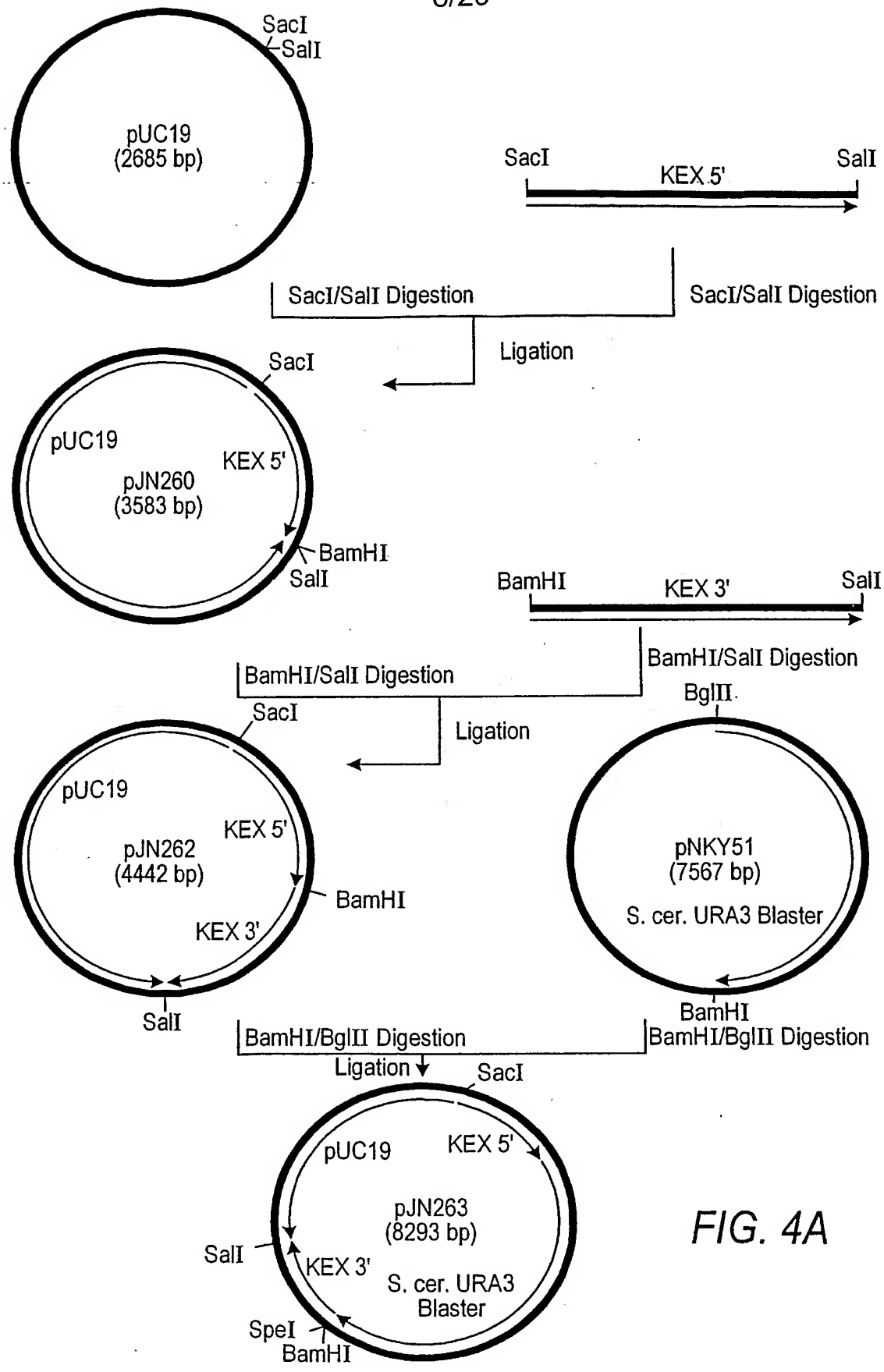

SacI
SalI
pUC19
(2685 bp)
SacI
KEX 5'
SalI
SacI/SalI Digestion
SacI/SalI Digestion
Ligation
SacI
pUC19
KEX 5'
pJN260
(3583 bp)
BamHI
SalI
BamHI
KEX 3'
SalI
BamHI/SalI Digestion
BamHI/SalI Digestion
BglII
SacI
Ligation
pUC19
KEX 5'
pJN262
(4442 bp)
BamHI
KEX 3'
SalI
pNKY51
(7567 bp)
S. cer. URA3 Blaster
BamHI
BamHI/BglII Digestion
BamHI/BglII Digestion
Ligation
SacI
pUC19
KEX 5'
pJN263
(8293 bp)
SalI
KEX 3'
S. cer. URA3
Blaster
SpeI
BamHI

FIG. 4A

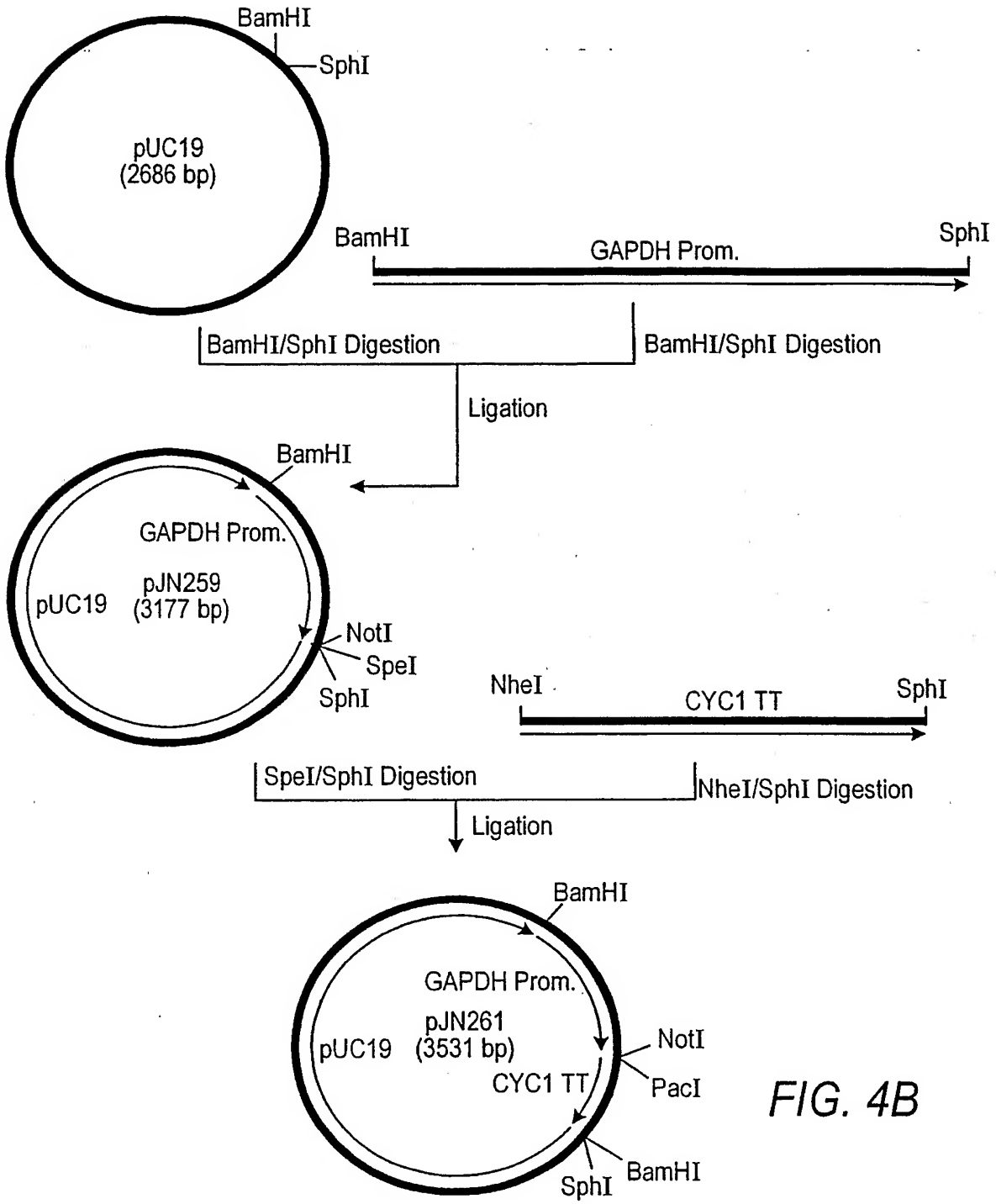

BamHI
SphI
pUC19
(2686 bp)
BamHI
GAPDH Prom.
SphI
BamHI/SphI Digestion
BamHI/SphI Digestion
Ligation
BamHI
GAPDH Prom.
pJN259
pUC19 (3177 bp)
NotI
SpeI
SphI
NheI
CYC1 TT
SphI
SpeI/SphI Digestion
NheI/SphI Digestion
Ligation
BamHI
GAPDH Prom.
pJN261
pUC19 (3531 bp)
CYC1 TT
NotI
PacI
SphI
BamHI

FIG. 4B

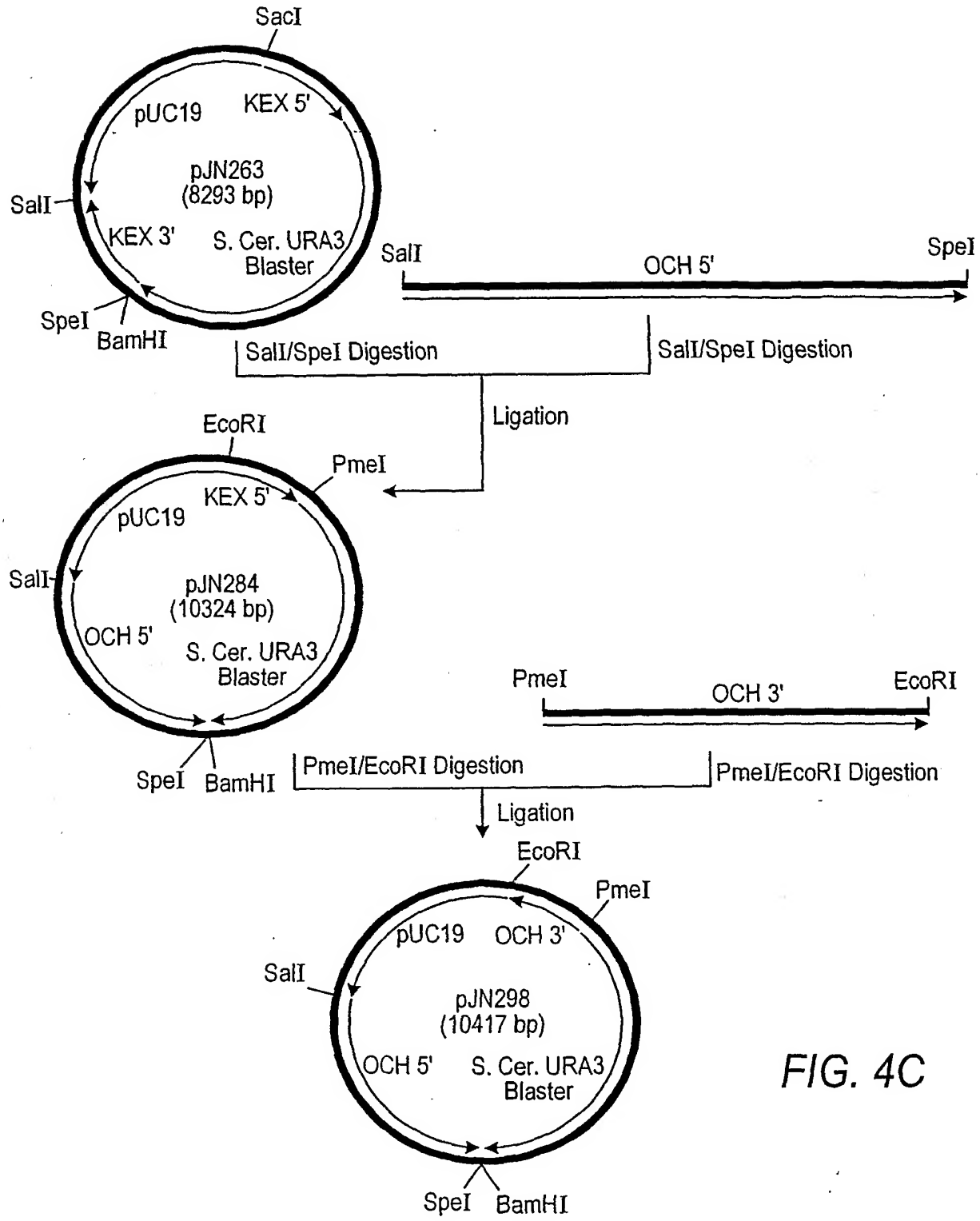

SacI
pUC19
KEX 5'
pJN263
(8293 bp)
SalI
KEX 3'
S. Cer. URA3
Blaster
SpeI
BamHI
SalI
OCH 5'
SpeI
SalI/SpeI Digestion
SalI/SpeI Digestion
Ligation
EcoRI
PmeI
KEX 5'
pUC19
SalI
pJN284
(10324 bp)
OCH 5'
S. Cer. URA3
Blaster
SpeI
BamHI
PmeI
OCH 3'
EcoRI
PmeI/EcoRI Digestion
PmeI/EcoRI Digestion
Ligation
EcoRI
PmeI
pUC19
OCH 3'
SalI
pJN298
(10417 bp)
OCH 5'
S. Cer. URA3
Blaster
SpeI
BamHI

FIG. 4C

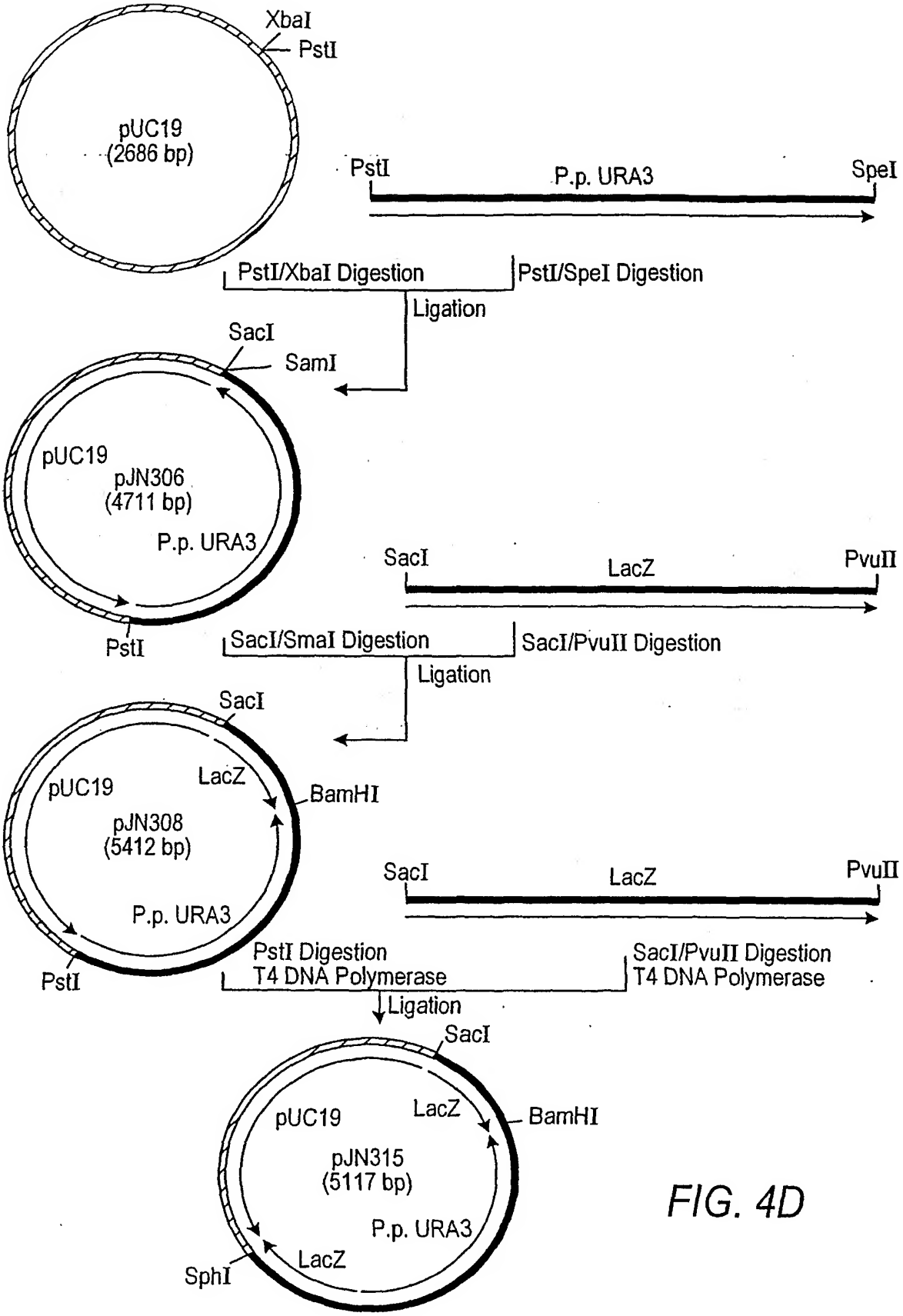

XbaI
PstI
pUC19
(2686 bp)
PstI
P.p. URA3
SpeI
PstI/XbaI Digestion
PstI/SpeI Digestion
Ligation
SacI
SamI
pUC19
pJN306
(4711 bp)
P.p. URA3
PstI
SacI
LacZ
PvuII
SacI/SmaI Digestion
SacI/PvuII Digestion
Ligation
SacI
LacZ
pUC19
BamHI
pJN308
(5412 bp)
P.p. URA3
PstI
SacI
LacZ
PvuII
PstI Digestion
T4 DNA Polymerase
SacI/PvuII Digestion
T4 DNA Polymerase
Ligation
SacI
LacZ
pUC19
BamHI
pJN315
(5117 bp)
P.p. URA3
LacZ
SphI

FIG. 4D

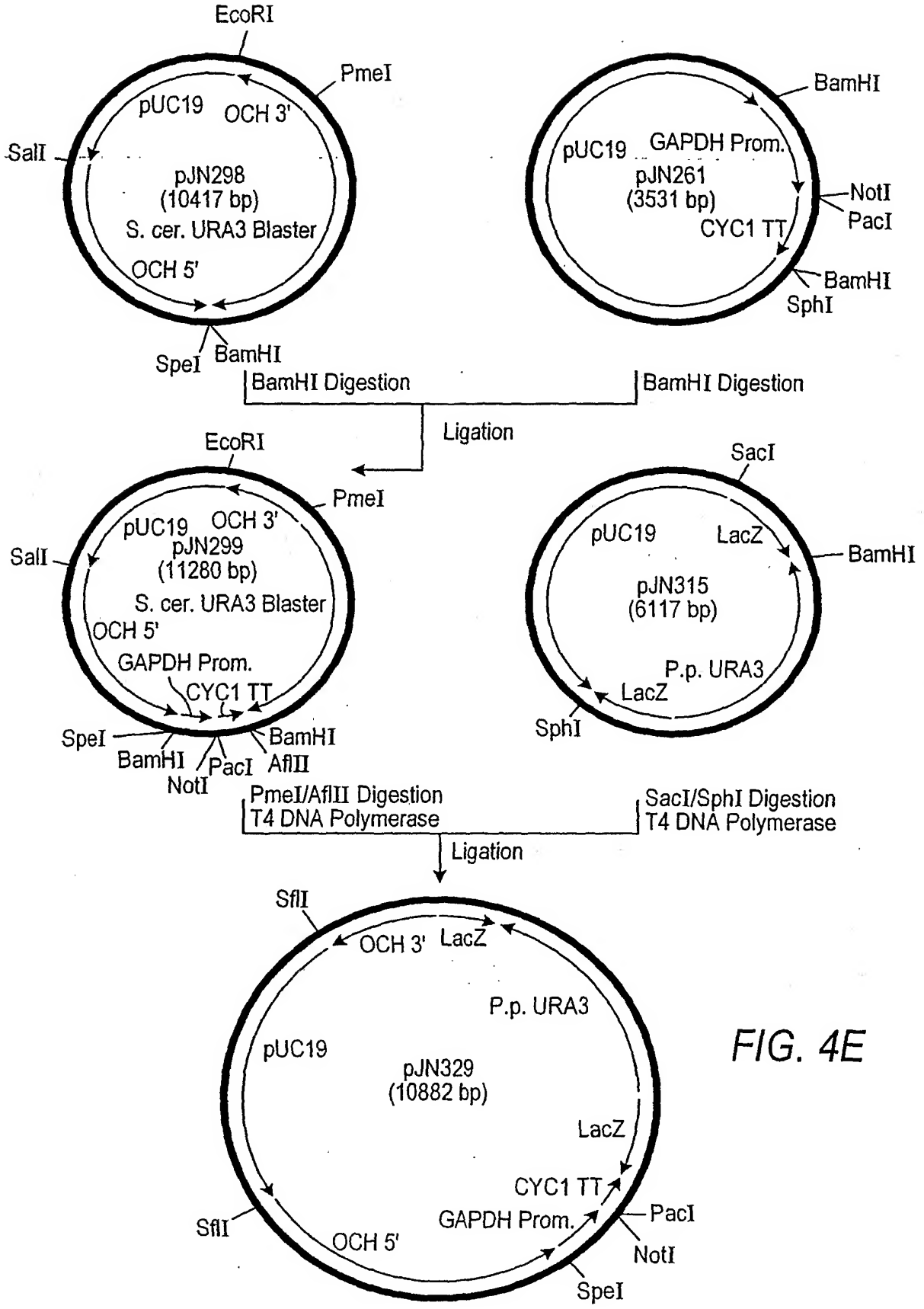

EcoRI
PmeI
pUC19
OCH 3'
SalI
pJN298
(10417 bp)
S. cer. URA3 Blaster
OCH 5'
SpeI
BamHI
BamHI
pUC19
GAPDH Prom.
pJN261
(3531 bp)
CYC1 TT
NotI
PacI
BamHI
SphI
BamHI Digestion
BamHI Digestion
Ligation
EcoRI
PmeI
pUC19
OCH 3'
SalI
pJN299
(11280 bp)
S. cer. URA3 Blaster
OCH 5'
GAPDH Prom.
CYC1 TT
SpeI
BamHI
BamHI
NotI
PacI
AflII
SacI
pUC19
LacZ
BamHI
pJN315
(6117 bp)
P.p. URA3
LacZ
SphI
PmeI/AflII Digestion
T4 DNA Polymerase
SacI/SphI Digestion
T4 DNA Polymerase
Ligation
SfiI
OCH 3'
LacZ
P.p. URA3
pUC19
pJN329
(10882 bp)
LacZ
CYC1 TT
GAPDH Prom.
PacI
NotI
SpeI
SfiI
OCH 5'

FIG. 4E

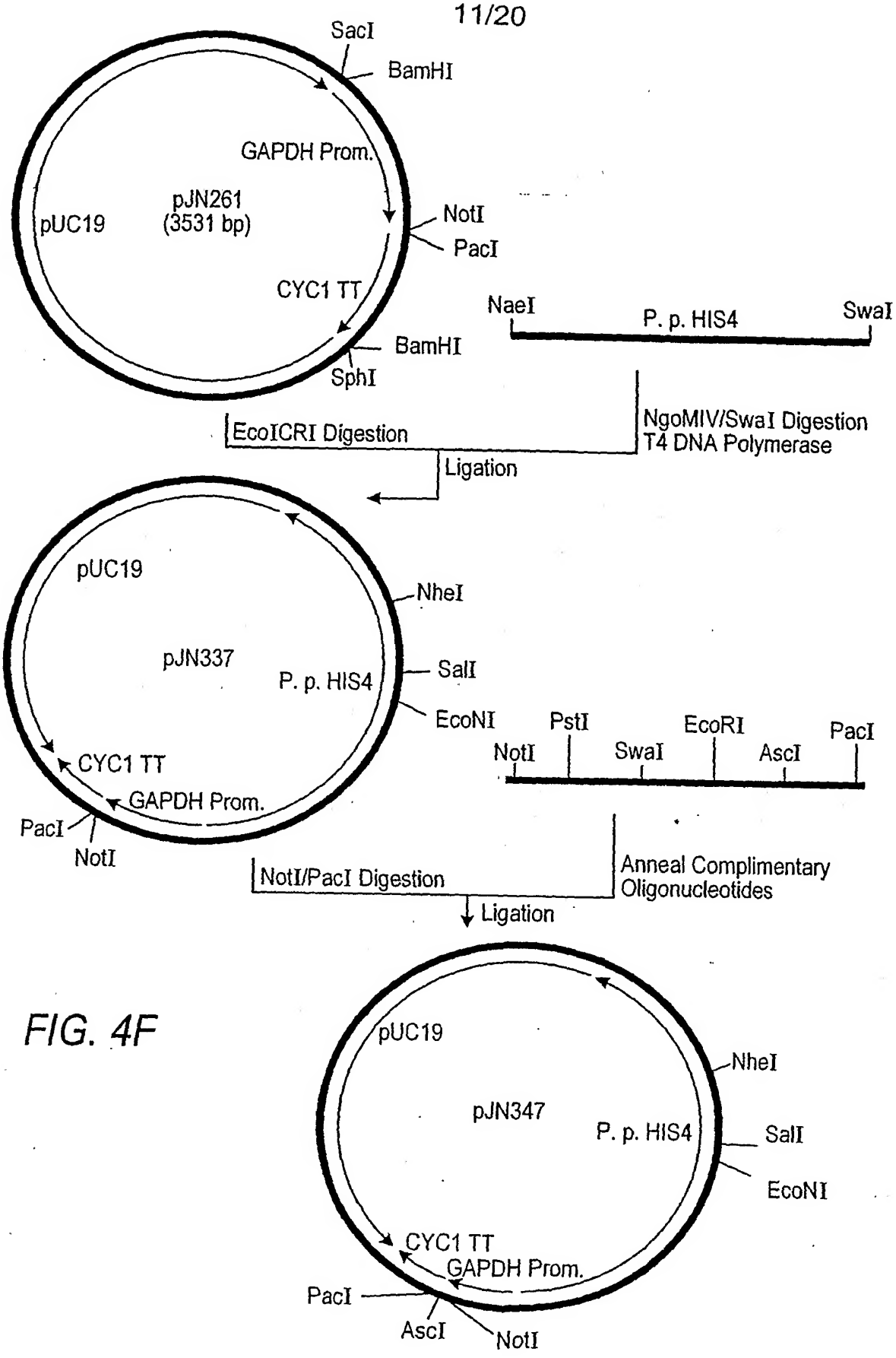

SacI
BamHI
GAPDH Prom.
pJN261
(3531 bp)
pUC19
NotI
PacI
CYC1 TT
BamHI
SphI
NaeI
P. p. HIS4
SwaI
EcoICRI Digestion
NgoMIV/SwaI Digestion
T4 DNA Polymerase
Ligation
pUC19
NheI
pJN337
SalI
P. p. HIS4
EcoNI
PstI
EcoRI
PacI
NotI
SwaI
AscI
CYC1 TT
GAPDH Prom.
PacI
NotI
NotI/PacI Digestion
Anneal Complimentary
Oligonucleotides
Ligation
pUC19
NheI
pJN347
SalI
P. p. HIS4
EcoNI
CYC1 TT
GAPDH Prom.
PacI
AscI
NotI

FIG. 4F

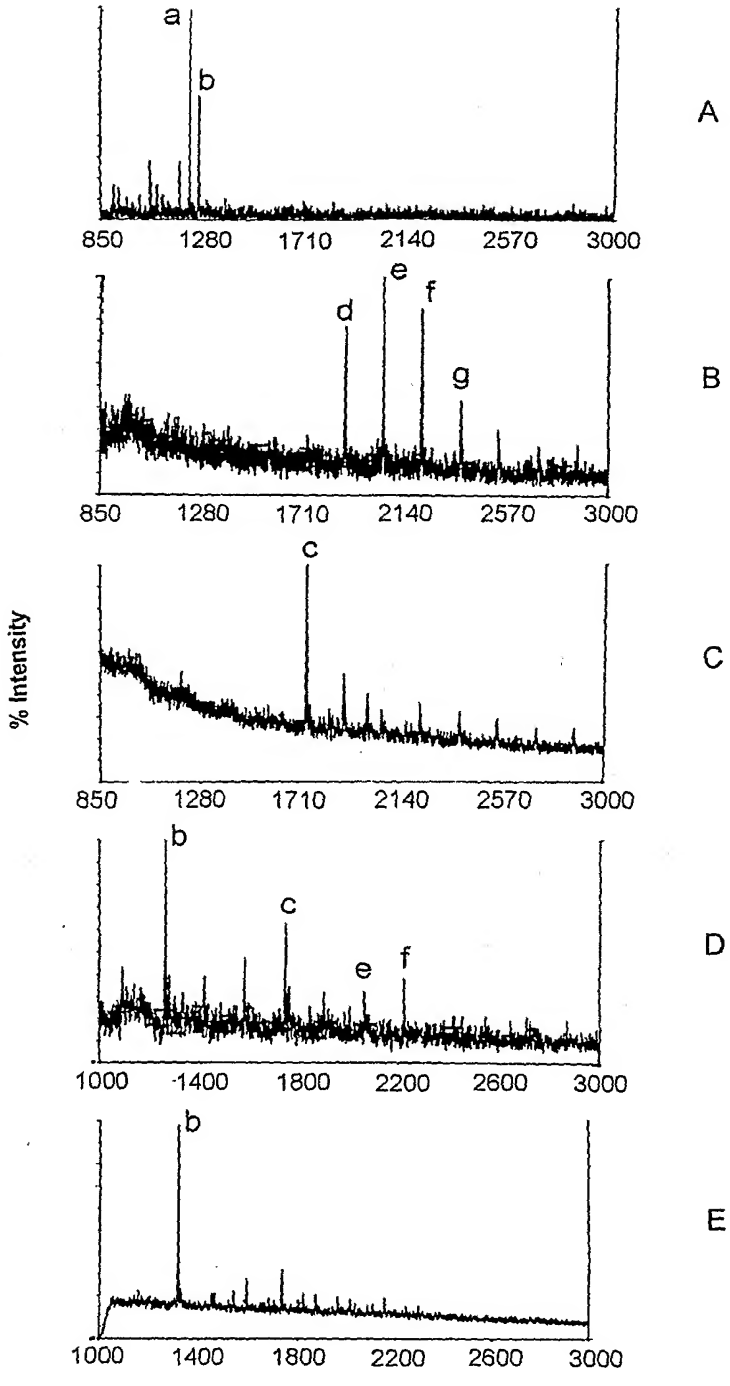
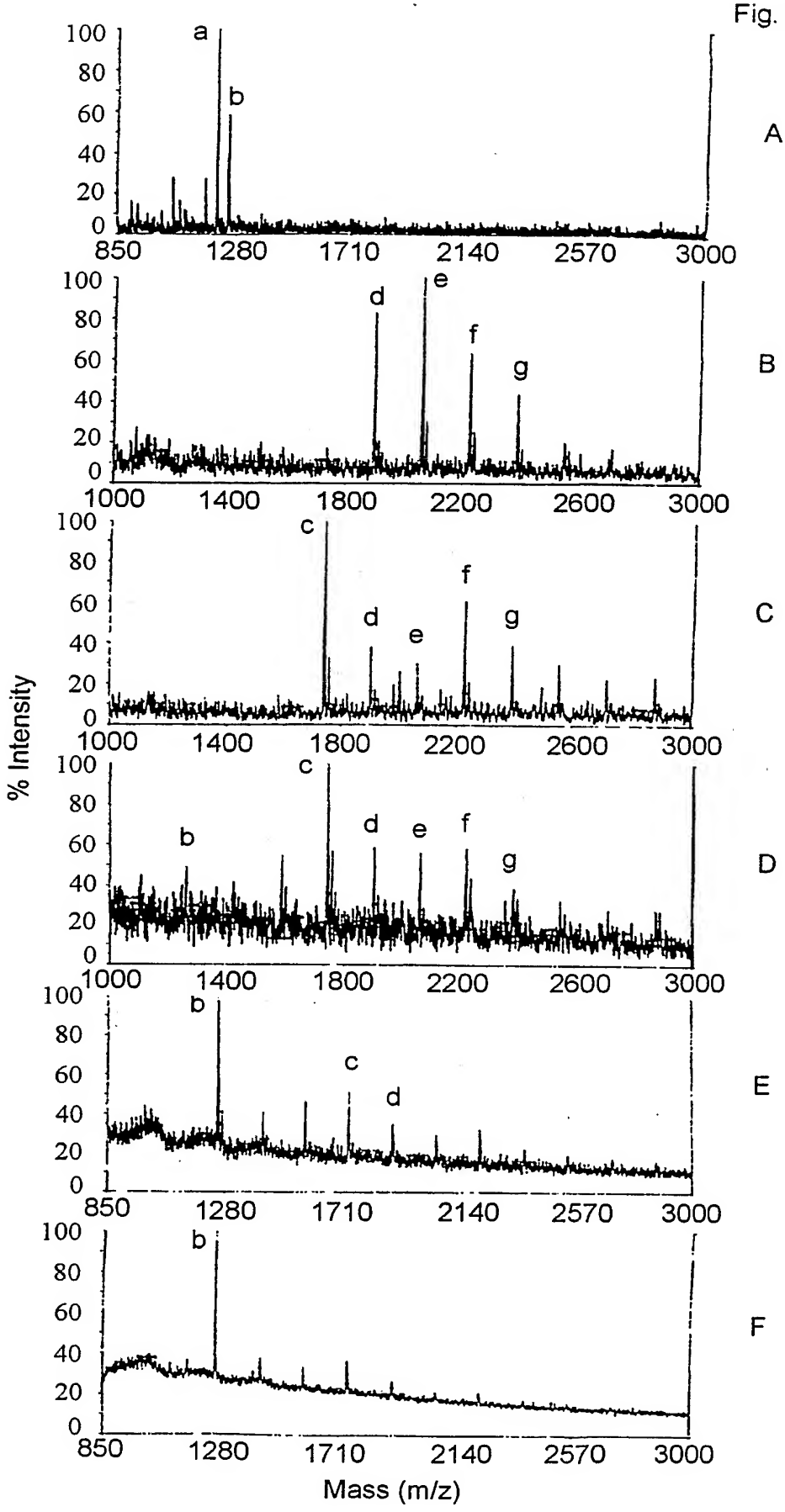

a
b
A
850
1280
1710
2140
2570
3000
e
f
d
g
B
850
1280
1710
2140
2570
3000
c
% Intensity
C
850
1280
1710
2140
2570
3000
b
c
e
f
D
1000
1400
1800
2200
2600
3000
b
E
1000
1400
1800
2200
2600
3000
Mass (m/z)

Fig. 5

Fig. 6


A
B
C
D
E
F
% Intensity
Mass (m/z)
100
80
60
40
20
0
850
1280
1710
2140
2570
3000
1000
1400
1800
2200
2600
a
b
c
d
e
f
g

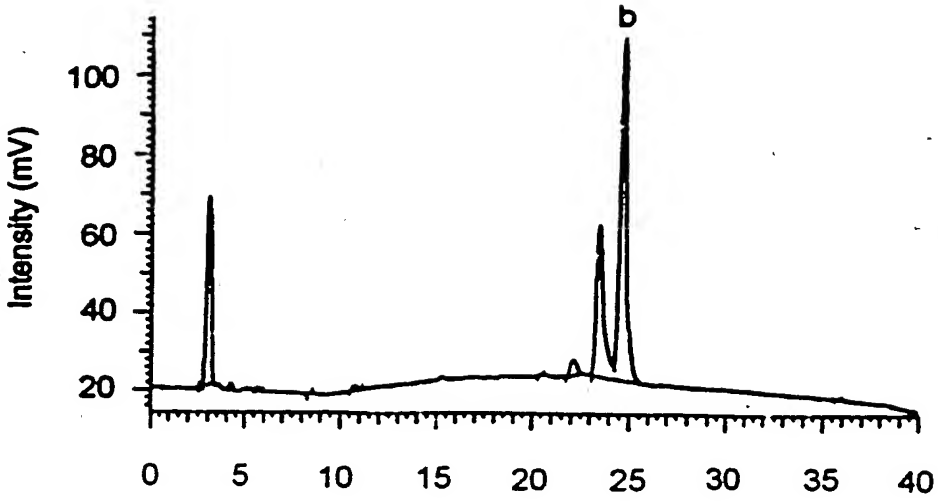


FIG. 7A

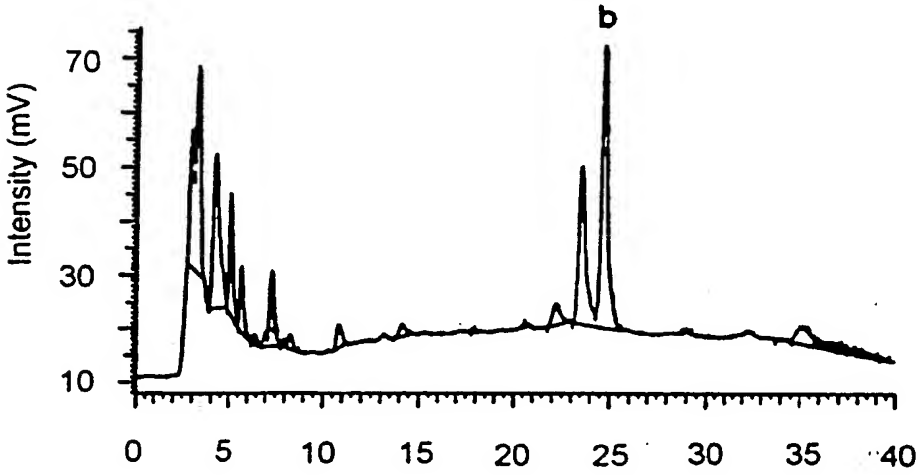


FIG. 7B

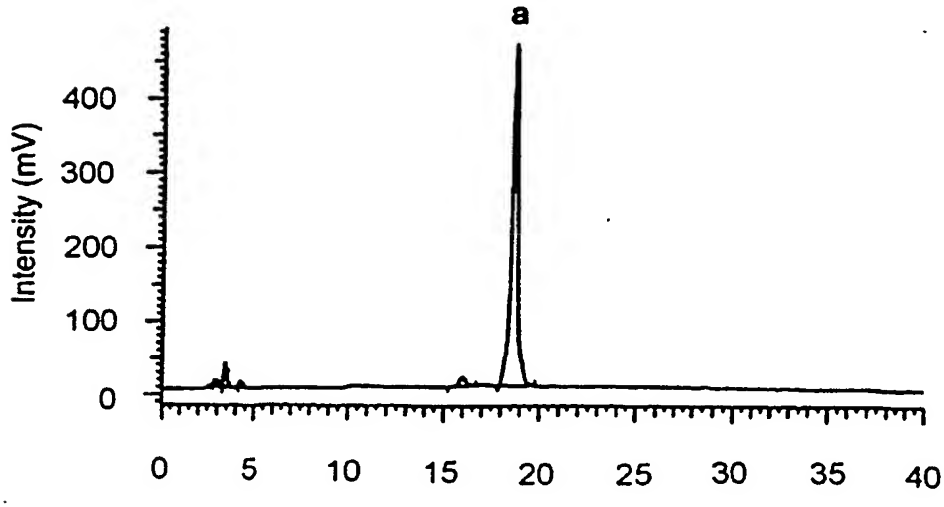


FIG. 7C

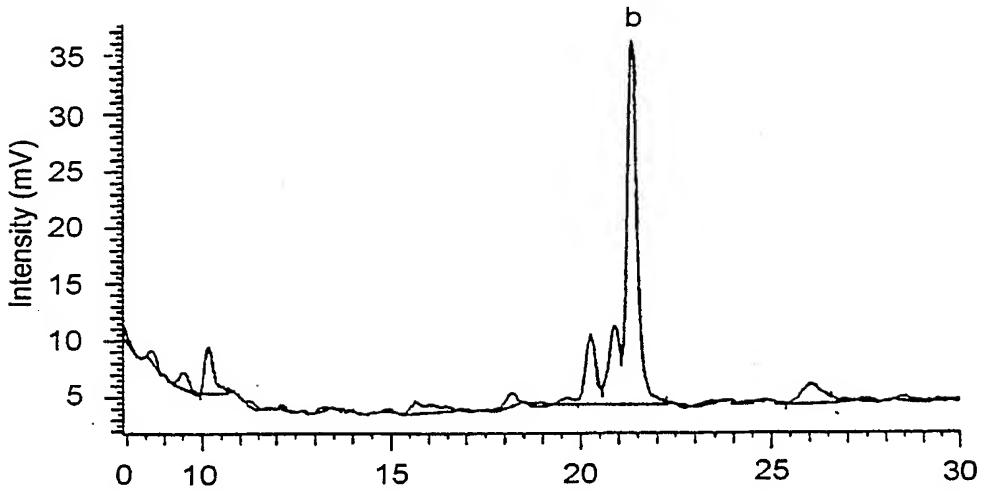


FIG. 8A

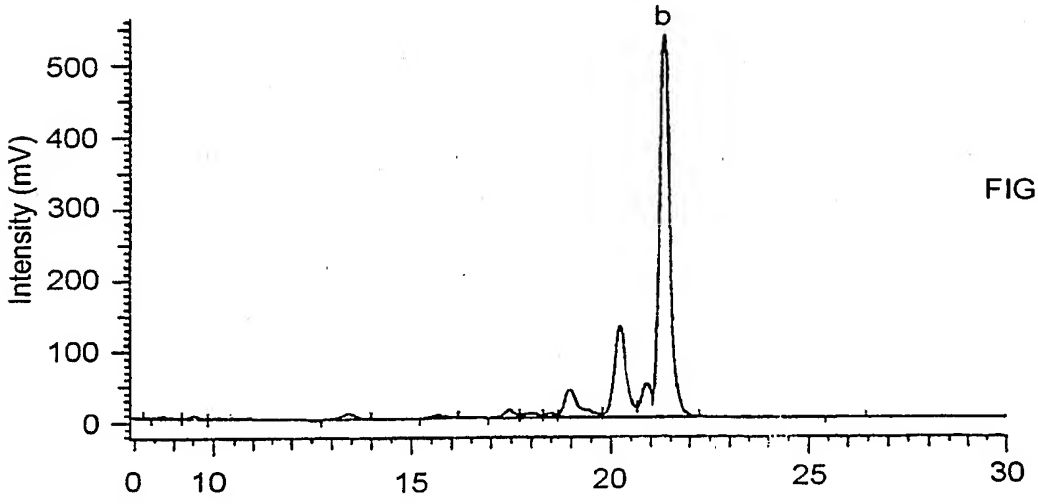


FIG. 8B

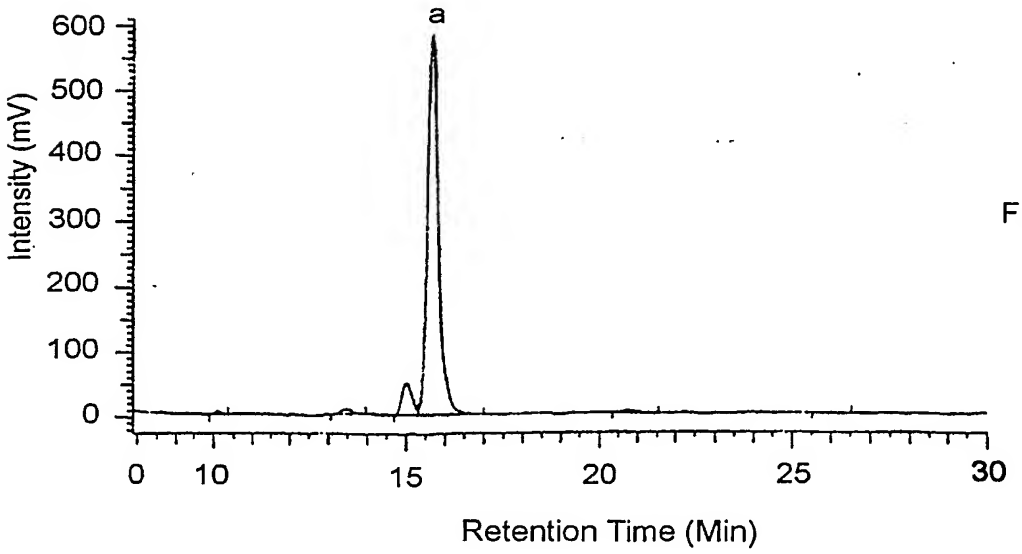


FIG. 8C

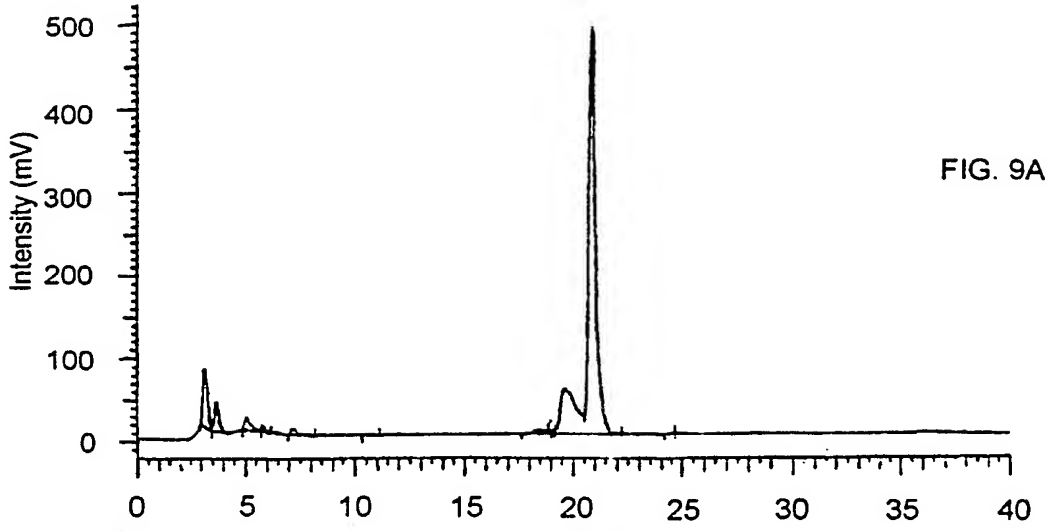


FIG. 9A

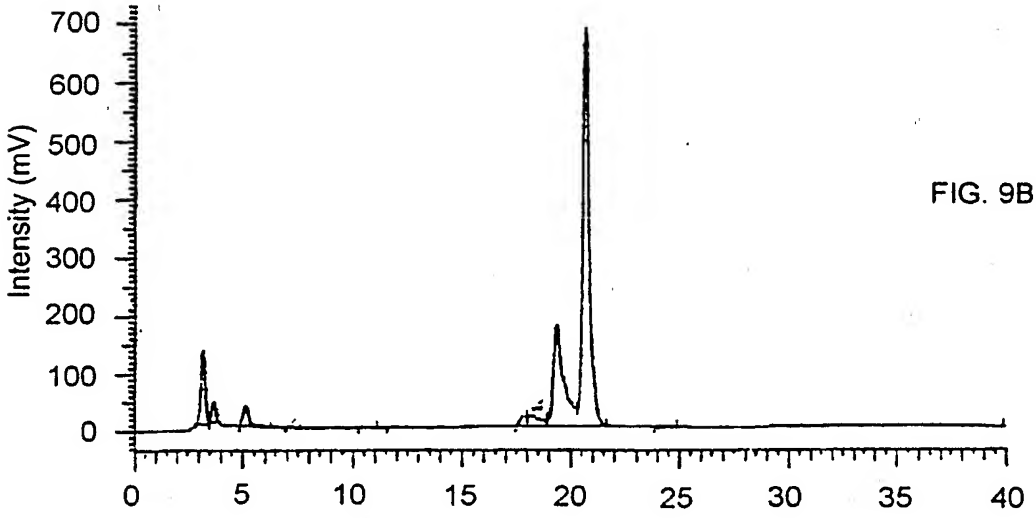


FIG. 9B

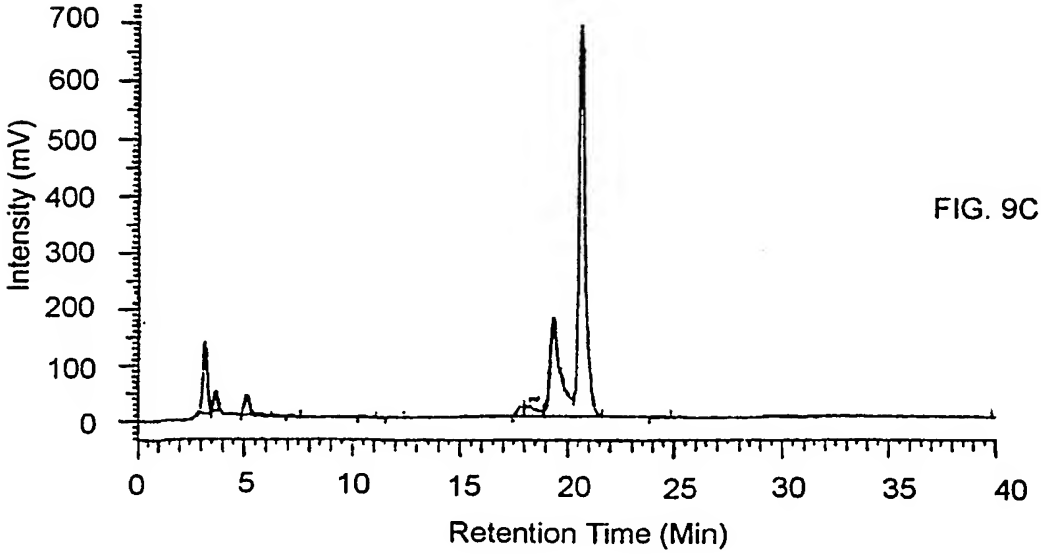


FIG. 9C

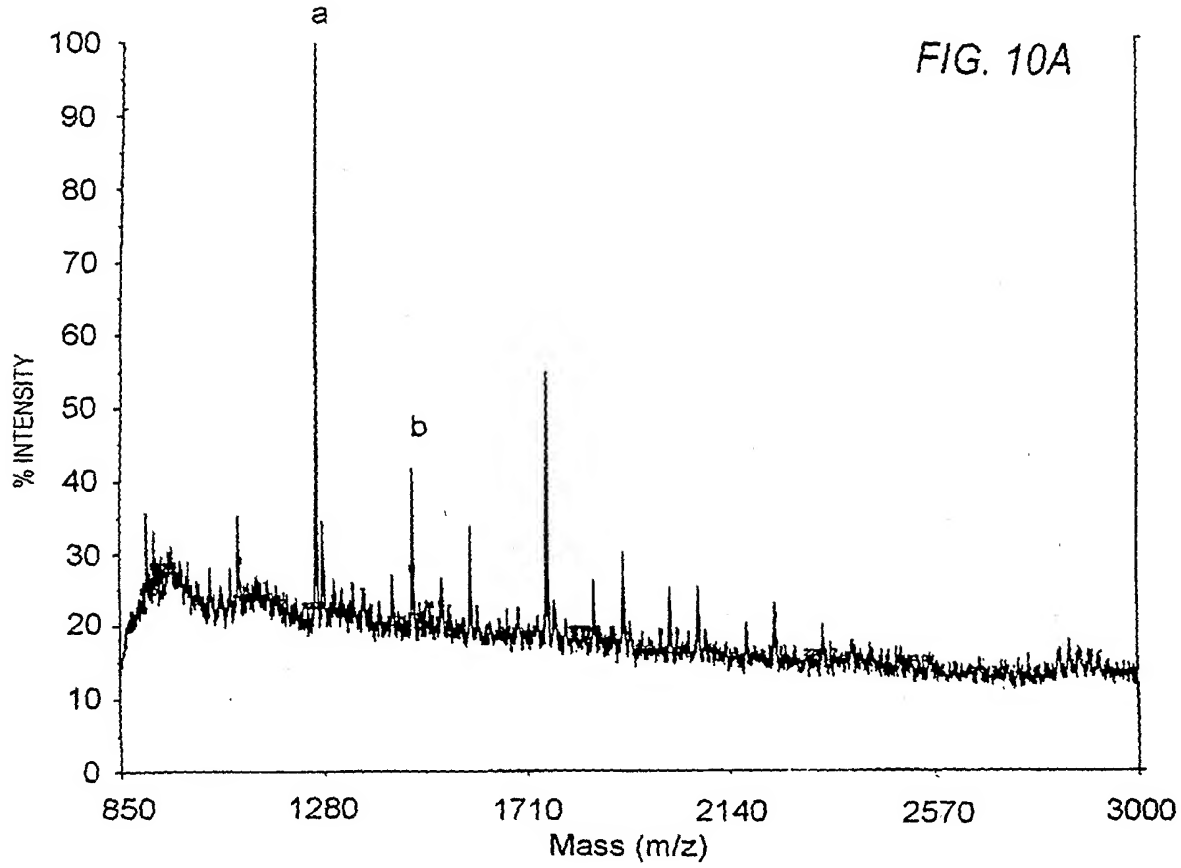


FIG. 10A

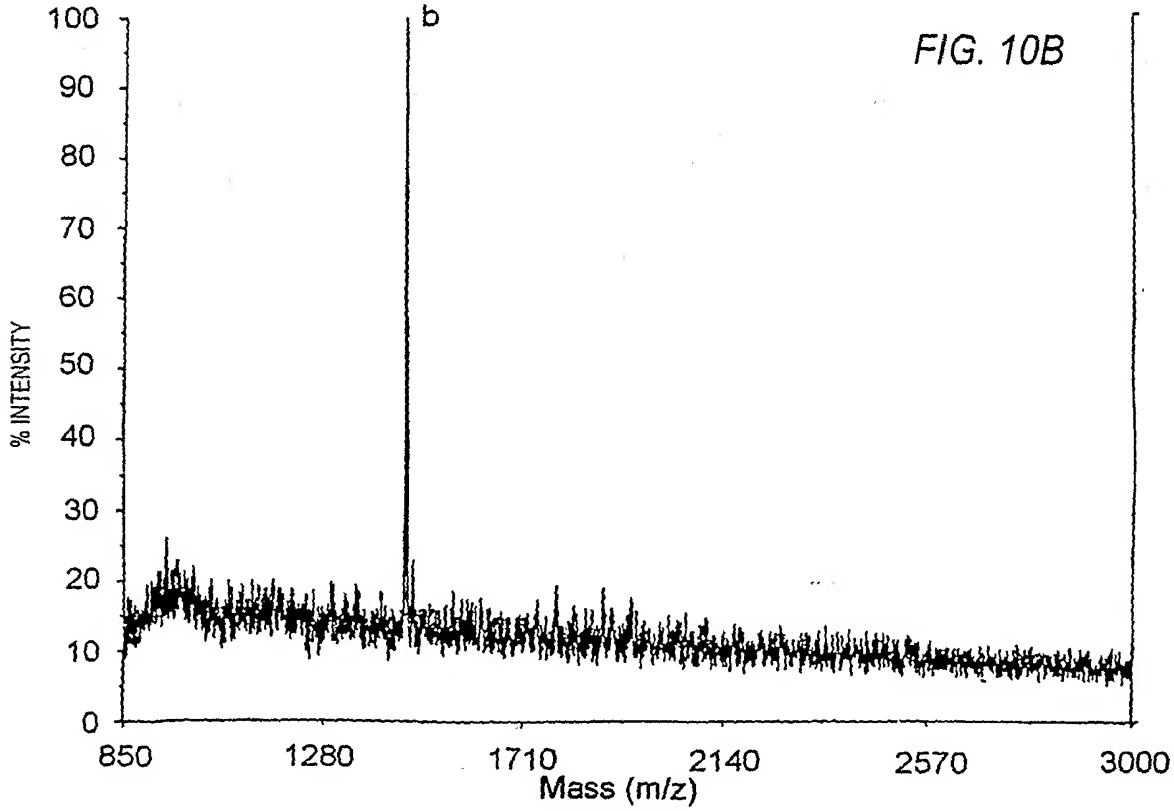
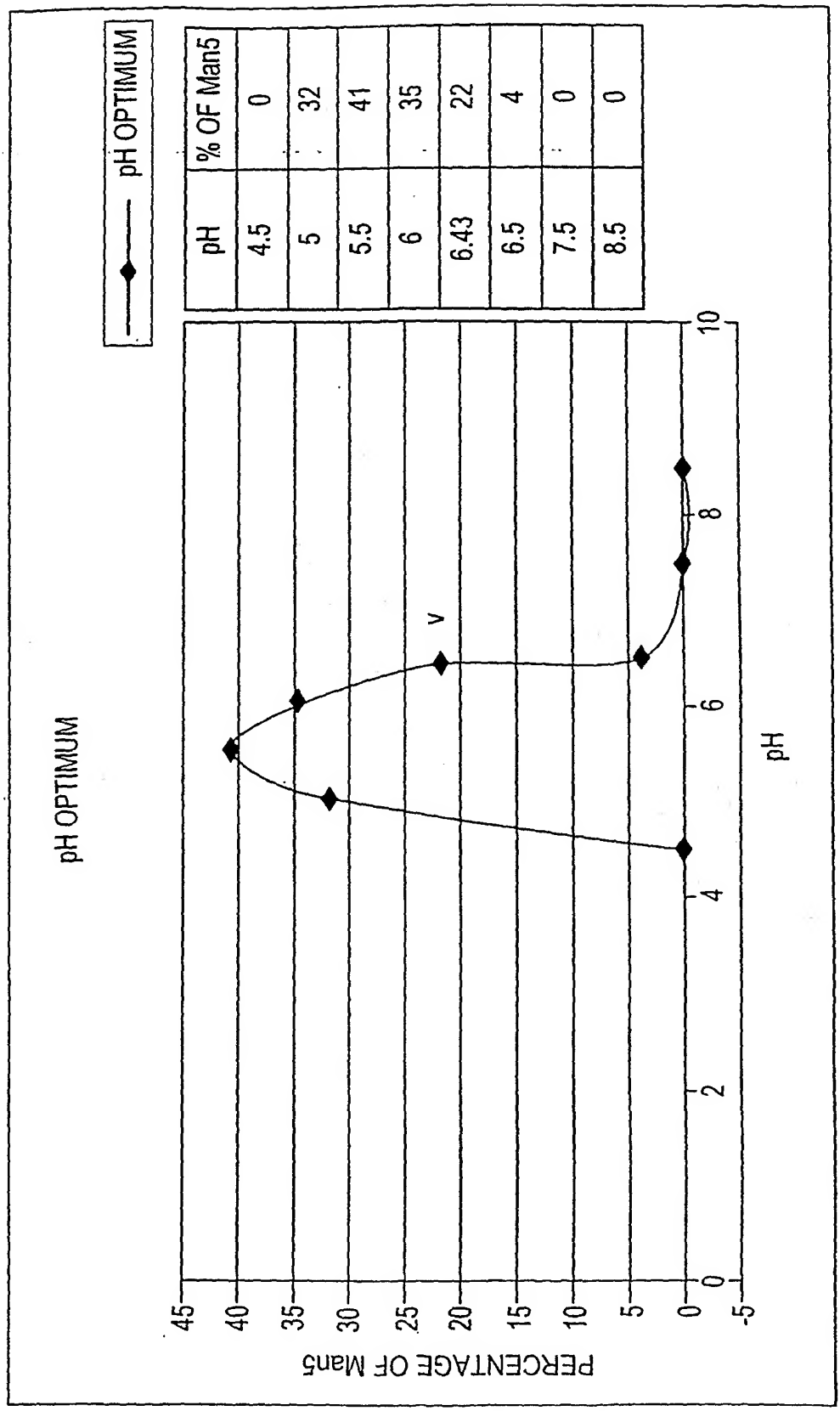


FIG. 10B



pH	% OF Man5
4.5	0
5	32
5.5	41
6	35
6.43	22
6.5	4
7.5	0
8.5	0

FIG. 11

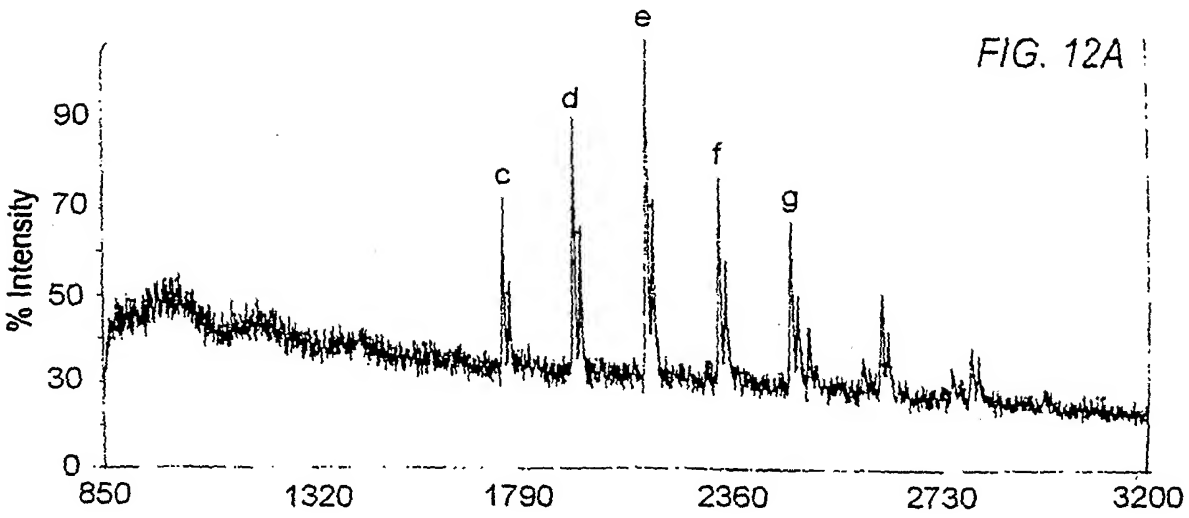


FIG. 12A

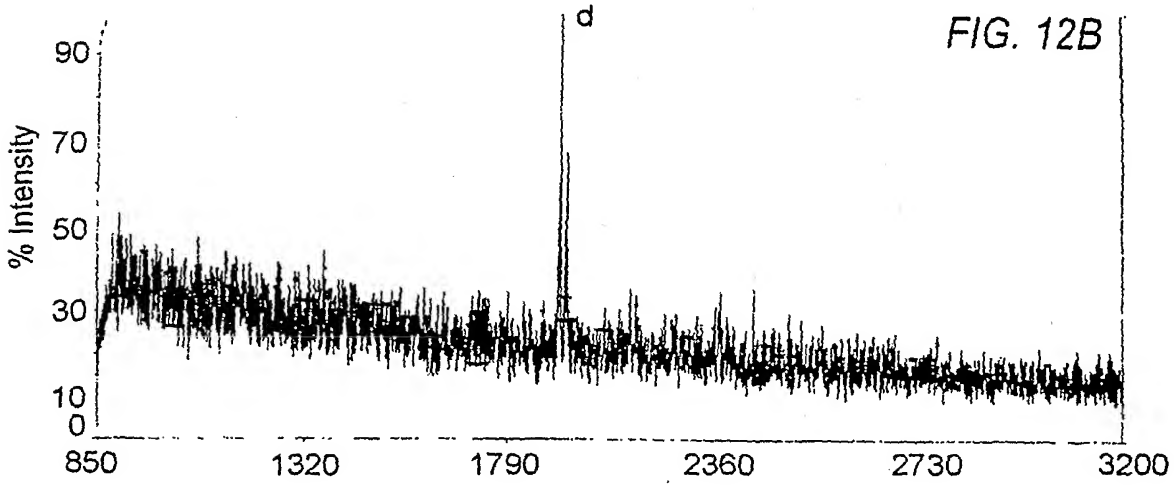


FIG. 12B

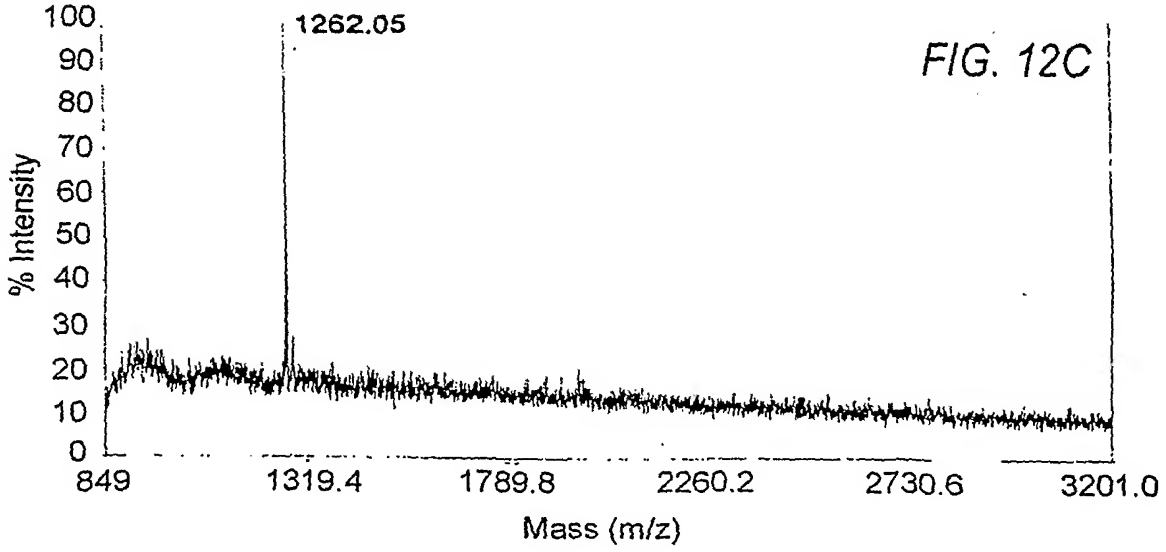


FIG. 12C

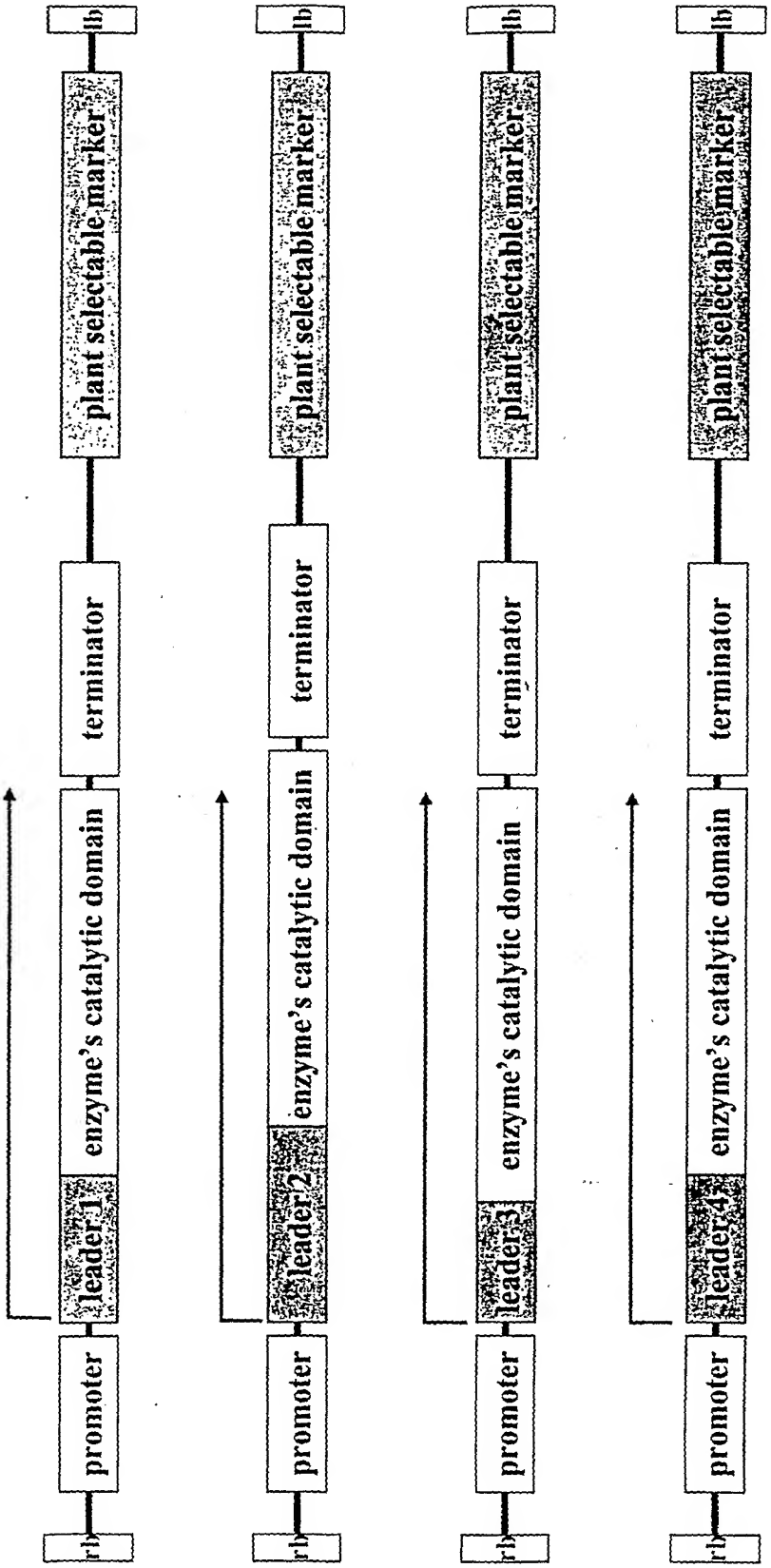

rb
promoter
leader1
enzyme's catalytic domain
terminator
plant selectable marker
lb
rb
promoter
leader2
enzyme's catalytic domain
terminator
plant selectable marker
lb
rb
promoter
leader3
enzyme's catalytic domain
terminator
plant selectable marker
lb
rb
promoter
leader4
enzyme's catalytic domain
terminator
plant selectable marker
lb

FIG.13